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ON THE SIMILARITY OF CHAIN LENGTH OF NUCLEIC ACIDS

By Ping-yao Cheng

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Communicated by John H. Northrop, September 8, 1959

In a communication two years ago, the author suggested that many cellular or viral ribonucleoproteins[1] (RNP) contain one molecule of ribonucleic acid (RNA) per particle, and that the molecular weight of the RNA in RNP particles is probably about 1.7×10^6 in many cases.[2] This suggestion was based on two experimental findings: (1) In the plant and animal viruses so far studied, the average weight of the total RNA is approximately 2×10^6 times the weight of a hydrogen atom per particle of virus.[3] Similar calculations[2] from data available for a class of large cellular RNP particles, 80S particles, from yeast, pea seedlings, and rat liver give a figure[4] of 1.7×10^6. (2) Determination of the molecular weight of the RNA from a rod-shaped virus, tobacco mosaic virus (TMV), consistently gave a value of 1.7×10^6 under a variety of preparative conditions.[5] The second finding has recently been extended to show that molecules of approximately this weight are also present in all the cellular RNA preparations, so far studied. These include diversified sources such as tobacco leaves,[6] mouse brain,[7] nuclei from calf thymus,[8] and microsomal particles from rat liver.[6]

In this communication, molecular weight of the RNA from a spherical virus, turnip yellow mosaic virus (TYMV), is reported. These values reported for the molecular weights of RNA[9] are then compared with those of deoxyribonucleic acids (DNA) from various sources, and the regularity revealed by this comparison is discussed.

TYMV RNA.—The RNA was isolated at 3°C from this spherical plant virus by a modification of the Gierer-Schramm procedure.[10] A highly purified TYMV suspension[11] containing 1.88 mg virus per ml of 0.01 *M* sodium phosphate, pH 7.2, was shaken for three minutes with an equal volume of 80 per cent redistilled phenol in the phosphate buffer. The resulting emulsion was centrifuged to separate the phases. The aqueous phase was removed and subjected to the process once more. The final clear aqueous layer was extracted with three volumes of ether five times, and then bubbled with N_2 to remove the residual ether. The preparation contained 76 per cent of the RNA in the initial suspension of virus.

A typical schlieren pattern, which was obtained immediately after the preparation was made, is shown in Figure 1. In contrast to the reported sedimentation diagrams for the RNA isolated by a similar procedure from TMV[12] and all cellular sources,[6–8] it is seen to contain a single and rather homogeneous component. The

RNA has a molecular weight of about 1.7×10^6. This is in agreement with the weight of RNA per TYMV particle.[13] Hence, there is one molecule of RNA per TYMV particle. The validity of the author's suggestion has, therefore, been shown for a spherical virus, a rod-shaped virus, and microsomal particles.

The present preparation of TYMV RNA is unstable and dissociates even upon storage at 5°C. The same is true of all RNA samples prepared from other sources by a similar procedure. This instability will explain the much lower values of molecular weight which were previously reported by other workers.[14, 15]

DNA from Bacteriophage φX174.—A very interesting discovery has been recently made by Sinsheimer.[16] The molecular weight of the DNA isolated from bacterial virus φX174 is 1.7×10^6, and this value is also the weight of DNA per φX particle. It would be surprising if this situation were limited to this bacterial virus alone. In comparison with the results on RNP described above, the suggestion for RNP is generalized to include deoxyribonucleoprotein (DNP) as follows: A RNP or DNP particle containing about 1.7×10^6 gm of nucleic acid per mole very likely has only one molecule of the nucleic acid.

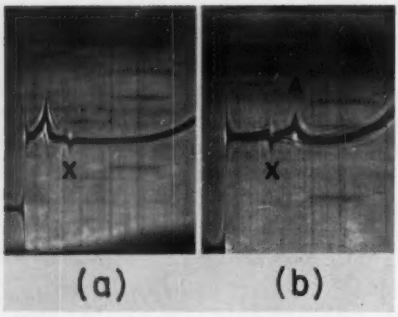

FIG. 1.—Schlieren sedimentation diagram of TYMV RNA (1 mg/ml) in 0.01 *M* sodium phosphate, pH 7.2, at 2°C. Pictures (a) and (b) taken after 4 and 18 min at 59,780 rpm, respectively. The $s_{20,w}$ of the RNA boundary, A, is 28.28 at this concentration (X is caused by a scratch mark on the cell wall).

Cellular DNA.—The DNA preparations from calf thymus, obtained by various procedures, were heterogeneous. The DNA molecule with a molecular weight of 4×10^6 has the highest frequency of occurrence.[17] The analysis on the distribution of molecular weight has not been done yet on the DNA preparations from other sources; however, it has been concluded that the DNA preparations from all diversified sources such as trout sperm, *E. coli*, fowl erythrocyte, and avian tubercle bacilli have the same average molecular weight as that from calf thymus.[18] This conclusion suggests that the DNA which most frequently occurs in many sources other than calf thymus also has a molecular weight of about 4×10^6. Very recently, the same molecular weight has been obtained for a rather homogeneous preparation of transforming principle from pneumococcus.[19]

Results of physicochemical studies indicate that the DNA particles from φX174[16] and all cellular[6] and viral[12] RNA so far studied are single-stranded. On the other hand, particles of all other DNA investigated are double-stranded. Hence, the experimental results described indicate that particle weights of about 1.7×10^6 and 4×10^6 have been found at the highest frequency for the single-stranded and double-stranded nucleic acids respectively. In view of the uncertainty involved in the molecular weight values, due to either heterogeneity of the sample studied or approximation of molecular model employed in the calculation, or both, the former figure cannot be considered as different from one-half of the latter. That is, chains of all these nucleic acids appear to have the same length of about 5,000 nucleotides. It does not matter whether the nucleic acid is RNA or DNA. Neither does it

make a difference if the chains are present in the single-stranded or double-stranded form. This has been found true for the preparations from viruses, and from cells of plant, animal, or bacterial origin. The only exception so far is the reported value of 13×10^6 for the molecular weight of DNA from bacteriophage T_4,[20] which is known to have an unusual chemical composition.

In view of the fact that the preparations were obtained with a variety of quite different procedures and that preparations such as transforming principles and viral RNA manifest biological activity, it seems likely that the similarity of chain length described exists among viral and cellular nucleic acids *in situ*.

The term "chain" of nucleic acid used here should not be construed to imply that all these 5,000 nucleotides are linked together only by phosphodiester bonds. The experimental data needed to settle this question are not available at present.

Discussion.—The similarity of chain length suggests that there widely occurs a mechanism in cells for controlling the length of nucleic acid chains. The wide occurrence indicates that it is inadequate to consider the base composition and base sequence of the nucleic acids responsible for the control. One can envisage the existence inside a cell of some kind of "surface" on which the chains are made from nucleotide blocks and by which the chain length is controlled. The larger groove on the two-stranded structure of Watson and Crick for DNA could be a "surface" of this category.[21] At present, however, there is no convincing explanation of why the "surface" should not be of some material other than DNA or why DNA and RNA should use the same kind of "surface."

It is interesting to note that these nucleic acids appear to have a function in common. They are suggested to control the within-cell synthesis of specific viral or cellular proteins. In the case of viral RNA, Fraenkel-Conrat[22] infected the plant with TMV which was made by using the RNA of one strain and the protein of another, and he found that the protein of the viral progeny resembled closely that of the strain from which the RNA was taken but had little resemblance to that of the other parental strain. Furthermore, evidence has been presented that RNA from many plant and animal viruses are infectious,[23] although in the case of the animal viruses much of the evidence is weak owing to lack of proper controls in the experiments. That DNA from the ϕX virus is infectious has not been demonstrated. There is little doubt, however, that the nucleic acid of all viruses plays a genetic role similar to that in TMV.

As to the cellular nucleic acids, the RNA in microsomal particles has been widely inferred to be templates for protein synthesis in the cytoplasm. It is likely that the DNA of human spermatozoa controls, at least in part, the amino acid sequence of haemoglobin.[24] Transforming principles from pneumococcus control the syntheses of the enzymes, mannitol phosphate dehydrogenase,[25] and amylomaltase.[26] Our knowledge about nucleus RNA in this respect is lacking. It will be interesting to see whether the high molecular weight nucleus RNA acts as templates for the protein synthesis in the nucleus, or as the precursor of the high molecular weight cytoplasmic RNA, or as both.

[1] The term nucleoprotein is used throughout the text to denote also particles containing substances in addition to nucleic acid and protein.

[2] Cheng, P., *Nature*, **179**, 426 (1957).

[3] Frisch-Niggemeyer, W., *Nature*, **178**, 308 (1956).

[4] This figure was recently given for 70S particles from *E. coli* also. Tissieres, A., and J. D. Watson, *Nature*, **182**, 778 (1958).

[5] Hopkins, G. R., and R. L. Sinsheimer, *Biochem. Biophys. Acta*, **17**, 476 (1955).

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[7] Cheng, P., *Biochem. Biophys. Acta* (in press).

[8] Cheng, P., *Nature*, **184**, 190 (1959).

[9] *Soluble* RNA will not concern us here.

[10] Gierer, A., and G. Schramm, *Nature*, **177**, 702 (1956).

[11] Was kindly supplied by Professor C. A. Knight of Virus Laboratories at the University of California, Berkeley.

[12] Gierer, A., *Zeit. Naturf.*, **13B**, 477 (1958).

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[14] Markham, R., and J. D. Smith, *Biochem. J.*, **52**, 565 (1952).

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[18] Sadron, Ch., J. Pouyet, and R. Vendrely, *Nature*, **179**, 263 (1957).

[19] Roger, M., personal communication.

[20] Meselson, M., *Cold Spring Harbor Symposia Quant. Biol.*, **23**, 11 (1958).

[21] Cf. Felsenfeld, G., D. R. Davies, and A. Rich, *J. Am. Chem. Soc.*, **79**, 2023 (1957).

[22] Fraenkel-Conrat, H., *J. Amer. Chem. Soc.*, **78**, 882 (1956).

[23] For instance, reference 10.

[24] Ingram, V. M., *Nature*, **180**, 326 (1957).

[25] Marmur, J., and R. D. Hotchkiss, *J. Biol. Chem.*, **214**, 383 (1955).

[26] Lacks, S., and R. D. Hotchkiss, personal communication.

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*THE EFFECT OF HYDRODYNAMIC SHEAR ON THE DEOXYRIBONUCLEIC ACID FROM T_2 AND T_4 BACTERIOPHAGES**

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Communicated by Francis O. Schmitt, September 28, 1959

In 1956, Levinthal[1] reported autoradiographic studies of T_2 and T_4 bacteriophage deoxyribonucleic acid (DNA). He found that the DNA in each phage particle was present in the form of several chains, one of molecular weight about 45×10^6 and six or more of molecular weight about 12×10^6. Attempts to differentiate the large and the small pieces of phage DNA by ultracentrifugation have been unsuccessful. For example, in sedimentation velocity experiments Fleischman[2] found a single peak with a sedimentation coefficient of 30 to 35 S(vedbergs) (the lower value was found on deproteinized preparations); Meselson, Stahl, and Vinograd[3] reported the DNA to band with a Gaussian distribution, implying a uniform molecular weight, in equilibrium experiments in a cesium chloride gradient.

With the ultraviolet absorption system in the Spinco Model E ultracentrifuge, DNA solutions below 0.001 per cent concentration can be studied, and sedimentation coefficients ranging from 8–50 S have been reported.[4, 5] Calculating from the formula which Doty, McGill, and Rice[6] derived from studies on calf thymus DNA, the large and the small pieces of phage DNA could have sedimentation coefficients about 42 and 26 S. These values may not be accurate since they are deduced from an unwarranted extrapolation of the formula, and, moreover, recent studies have

shown that molecular weights and physical constants for DNA from different sources cannot be correlated;[7, 8] nevertheless, the failure to observe any complexity in the sedimentation diagrams has raised doubts about the *in vivo* existence of the "large piece," which could be an experimental artifact.

Thomas and Knight[9] have measured the sedimentation coefficient of the "large piece" using a partition centrifuge cell. They obtained a value of 40–58 S. The disparity between these and Fleischman's results is sufficient to provoke inquiry. The experiments reported below show that the investigations of Fleischman were probably made on DNA which had been inadvertently degraded. They also show that any physical experiment on phage DNA (and possibly DNA from other sources) must be attended by much greater manipulative precautions than have usually been employed.

Experimental.—Four bacteriophage samples were studied, of which two were given by Dr. H. Van Vunakis (T_2 and T_4), one by Dr. J. Tomizawa (T_2), and of which the fourth (T_2) was prepared by the author. Each preparation was finally purified by differential centrifugation until a clean translucent pellet was obtained. The phage were resuspended in neutral phosphate buffer at a suitable concentration (about 2×10^{12} phage per ml). Results from all these preparations were essentially identical.

Experiments were performed in a Spinco Model E ultracentrifuge using a cell with a 30 mm centerpiece. The optical density of the photographs was measured on a Joyce-Loebl microdensitometer. The density tracings were evaluated by the method of Schumaker and Schachman[5] to give the differential distribution curve. It should be emphasized that no attempt was made to bring the area under the different parts of the curves accurately into accord with the heights in the integral curves. The differential curve was drawn merely to give the best fit through the points derived from at least two of the optical density tracings.

Shear degradation:† Thomas[10] showed that the large piece of T_4 DNA was degraded during deproteinization, presumably by the shaking involved. Flow birefringence experiments on a shockate of T_2 phage demonstrated that some irreversible changes could be effected in the elongated DNA molecules by the hydrodynamic shear imposed by the Couette apparatus.[11] Both of these results suggested that hydrodynamic shear gradients of the same magnitude as those commonly imparted to solutions by such simple laboratory manipulation as stirring or pipetting could cause a reduction in the molecular weight of long DNA molecules.

To confirm these observations, T_2 or T_4 phage in 3 *M* cesium or sodium chloride were osmotically shocked (Anderson)[12] by the rapid addition of 20 volumes of 0.005 *M* versene pH 8.5, or cold water. Versene was employed in the case of phage preparations which had been treated with pancreatic deoxyribonuclease, to ensure that no enzymatic degradation occurred. Cesium or sodium chloride was then added to the shockate to molar concentration to minimize any association of protein and DNA through salt bonds; later it was found that these salts could be substituted by 0.2 *M* phosphate buffer. For many studies the DNA was banded in a cesium chloride gradient in a preparative centrifuge to free it from protein.[13] The DNA recovered from the band was diluted with water to lower the salt concentration and directly examined in the ultracentrifuge. No significant differences were detected between solutions treated in this way and the untreated shockates.

The solutions were sucked slowly into a syringe, a hypodermic needle was attached to the syringe, and the solutions were injected into the centrifuge cell at a controlled rate to subject them to a measurable average shear gradient. Figure 1 shows the effects of different shears. The mean shear rate for the passage through the hypodermic needle was calculated, assuming that the flow was streamline, from Kroepelin's formula[14]

$$\bar{G} = \frac{8V}{3\pi r^3 t}$$

where V is the volume passing in time t, and r is the radius of the capillary. (For example, forcing 1 ml through a No. 27 needle in 15 sec. imposes a mean shear gradient of 55,000 sec.$^{-1}$)

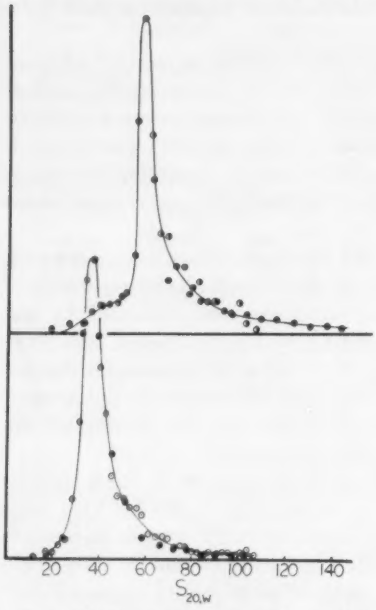

FIG. 1.—Distribution of sedimentation co-efficients in a 0.002% solution of DNA (recovered from banding a T_4 shockate in a cesium chloride gradient) in molar cesium chloride: (top) solution subjected to a shear gradient of 300 sec.$^{-1}$ during the filling of the cell; (bottom) solution subjected to 35,000 sec.$^{-1}$

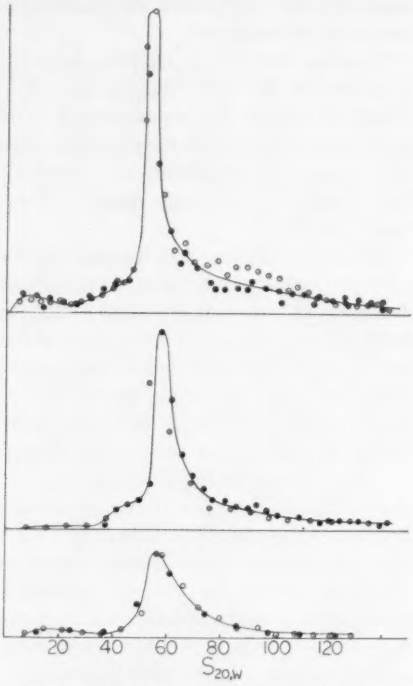

FIG. 2.—Distribution of sedimentation coefficients in a T_2 shockate in molar sodium chloride at the following DNA concentrations: (top) 0.0021%; (middle) 0.0014%; (bottom) 0.0007%.

The sedimentation diagram of the slowly loaded sample is typical of a large number of shockates examined. The addition of detergent or chymotrypsin to the solutions effected no obvious changes. The sedimentation coefficient of the main peak varied from 50–56 S in sodium chloride solutions, and from 60–70 S in cesium chloride solutions (the upper values in each case corresponded to the most dilute solutions examined). The main peak broadened noticeably as the concentration was lowered (Fig. 2).

The small peak or hump before the main peak varied in size and was assumed to indicate a small proportion of molecules ruptured in the turbulent solution at the moment of shocking. It rarely accounted for more than 10 per cent of the DNA and was frequently absent altogether. The long high molecular weight "tail" to the curve occasionally accounted for 25 per cent of the DNA, and, since its sedimentation coefficient was in some cases higher than that of the phage ghosts,[9] it was assumed that this DNA was associated and possibly crosslinked with protein. Support for this explanation was obtained from flow birefringence experiments indicating the presence of bonds which persisted despite the presence of strong salt in the solution, and which broke and reformed reversibly when the solution was briefly sheared in the apparatus. Further confirmation of the association of DNA and protein was afforded by the observation that from 25 per cent to 40 per cent of the DNA floated above the cesium chloride in the preparative banding experiments. Some of this DNA could be released by treatment with sodium dodecyl sulfate. The cause for the protein association is undetermined, but it is unlikely to be due to incomplete shocking since a plating assay revealed only 0.6 per cent survivors. Because only part of the DNA was free of protein the shockates could not be used to characterize the DNA of the phage, but they served to delimit the conditions under which the molecules were stable.

A series of experiments showed that when the titration of the solution was performed by a machine, a mean shear rate up to 25,000 sec.$^{-1}$ could be applied without any change in the sedimentation diagram. However, when the solution was injected by hand, even a shear rate as low as 16,000 sec.$^{-1}$ caused extensive breakdown. The integrity of the molecules could be safely preserved by injecting the solution slowly (1 ml/minute) by hand using a No. 22 needle (the largest the cell orifice will accommodate).

The difference between machine- and hand-pipetting might be explained by the plunger moving in a series of small jerks in the hand; however, a further feature of the sedimentation diagrams required explanation. If the flow in the needle is streamline, then the liquid through the center of the needle should suffer no shear gradient, and hence the sheared solution should show a mixture of degraded and undegraded molecules. In none of the five solutions examined was a bimodal curve detected. This could be understood if the rupture of the molecules occurred also in the turbulence at either end of the needle, where efficient mixing takes place and where high local shears could be present. Confirmation of this idea was obtained from another experiment in which half the contents of a cell was squirted through a fine needle; when this half of the solution was injected rapidly into the remainder of the solution in the cell, no undegraded molecules were detected, but when the sheared solution was added slowly to the unsheared half, a bimodal distribution with maxima at 29 and 53 S (in sodium chloride) was clearly resolved.

With the application of shears of the order of 20,000–40,000 sec.$^{-1}$ the DNA sedimented as a fairly symmetrical peak with a maximum about 30 S. However, by shearing more rapidly through a syringe needle the maximum could be dropped to 21 S; by 15 seconds' treatment in an Osterizer the maximum was dropped to 17 S.

No increase in the absorption of the DNA solutions at 260 mμ was detected accompanying the shear degradation.

Since the destructive shear in the case of turbulence cannot be evaluated, it is

difficult accurately to define the conditions which must be avoided to maintain the DNA intact. However, it is likely that any violent agitation of the solution, filtration, pipetting, even chromatography through a fine adsorbent could cause a rupture of the DNA chain.

In an attempt to demonstrate the DNA degradation which Thomas observed in deproteinization experiments,[10] a T_2 shockate was examined before and after gently shaking thirty times in a half-filled test-tube (Fig. 3). The presence of an immis-

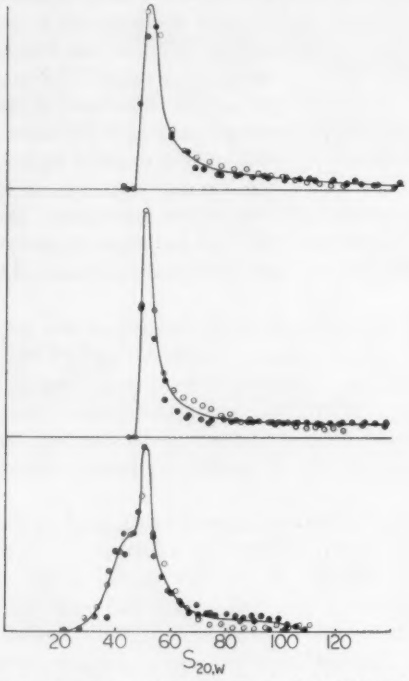

FIG. 3.—Effect of shaking T_2 shockate in 0.2 M pH 8 phosphate buffer (DNA concentration 0.001%): (top) untreated; (middle) shaken 30 times in test-tube; (bottom) shaken 30 times in test-tube in the presence of an equal volume of chloroform.

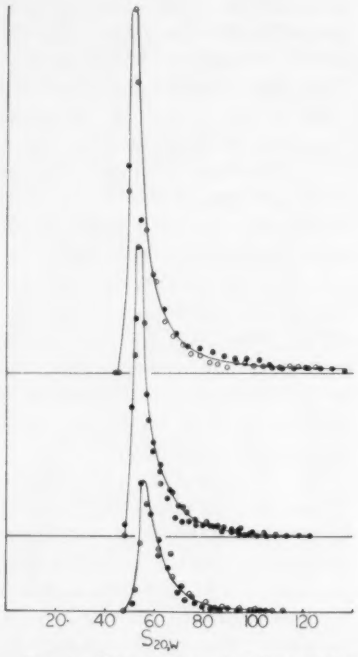

FIG. 4.—Distribution of sedimentation co-efficients in T_2 DNA released by treatment with pH 11 buffer. DNA concentrations: (top) 0.001%; (middle) 0.0007%; (bottom) 0.0004%.

cible liquid appears to be necessary for chain rupture to occur under these mild conditions of shaking.

Characterization of phage DNA: Since some of the DNA in the shockate appeared to be interacting with protein, methods were sought to prevent this interaction. Two methods are obvious: (*a*) the addition of excess competing molecules, or (*b*) raising the pH of the solution so that protein and DNA are similarly charged and mutually repel. Both methods were used and gave essentially similar diagrams, but the latter technique was the simpler, particularly when, on the advice of Dr. E. Freese, the osmotic shock treatment was omitted. The centrifuge cell was filled with borate or phosphate buffers of pH 11.0, and the requisite quantity of intact

phage was added from an Agla micrometer syringe. The DNA concentration was kept below 0.002 per cent to lessen the likelihood of concentration-dependent interactions. The solution was mixed by gentle inversion and allowed to stand for 10 minutes at room temperature to allow the phage to burst. The characteristic sedimentation patterns obtained in this manner are shown in Figure 4. The absence of the hump on the low S_{20} side confirmed that the presence of low S_{20} material in the shockates was adventitious.

The high S "tail" on the diagrams, which was much smaller than in the case of the shockates, demonstrated a lower tendency to associate with protein. Sedimentation equilibrium experiments showed that more than 80 per cent of the DNA could be banded at a specific gravity of 1.7; so it may be concluded that these sedimentation diagrams are representative of the DNA in phage.

In a few examples there has been a suggestion of a hump on the high S_{20} side at about 64 S (in sodium chloride). However, since minor irregularities are constantly present in the densitometer traces, it cannot be claimed that there is any reliable indication of heterogeneity in the DNA beyond an undoubted skewness in the peak—and that could be accounted for by a hypersharpening of the boundary.

The DNA released by high pH appeared to be at least as sensitive to shear as in the shockates.

Discussion.—Throughout this paper it has been assumed that the lowering of the sedimentation coefficient corresponds to a lowering of the molecular weight of the DNA. This assumption is reasonable since: (*a*) it has been shown that for homologous DNA samples the value of $S_{20,w}$ decreases with molecular weight,[6, 7] and (*b*) from Thomas's experiments, the treatment which destroys the large piece of DNA[10] also produces material with a lowered sedimentation coefficient. Moreover, it is difficult to imagine that the decrease in $S_{20,w}$ could come about as a result of a change of shape since the shearing of the molecules is accompanied by a decrease in the specific viscosity.

A possibility to be considered is that the changes brought about by shear do not represent a scission of the DNA double helix, but a dissociation of aggregates. If this is the case, the aggregates are probably specific, since they are present in DNA liberated by high pH or shocking, and they do not reform in the solutions after shearing. However, the possibility seems unlikely, because the sedimentation coefficients of the products drop as the violence of the shearing is increased, and there is no evidence for a stable sub-unit. It must be concluded that DNA can be degraded by hydrodynamic shear.

The relative fragility of the DNA demonstrated in these experiments is quite understandable on theoretical grounds. Frenkel[15] in 1944 deduced that long polymers might be degraded by shears of the order of a few thousand reciprocal seconds, and Bestul and Belcher,[16] for example, demonstrated the rupture of synthetic polymers of 500,000 molecular weight at shears of 60,000 $sec.^{-1}$. Since it is only too easy to apply shears (calculated for streamline flow) of 20–30,000 $sec.^{-1}$ in volumetric pipettes or hypodermic syringes, rupture of long-chain molecules is to be expected. Although DNA is a double-strand molecule, it is not easy to predict whether or not the molecule could resist a lengthwise stress twice as great as a single polynucleotide strand. Also it is not possible to calculate accurately the stress applied by the viscous forces to a molecule traversing the streamlines. For example, it is dubious if

Stokes's equation can be applied since the solvent molecules and hydrated ions are by no means small compared with the diameter of the DNA helix. However, Levinthal,[17] employing reasonable values for the bond strengths, calculated the critical shear rates to be about 10,000 sec.$^{-1}$. The observed threshold of about 25,000 sec.$^{-1}$ is in reasonable accord, if it can be assumed that the rupture occurs in streamline flow. The degradation of DNA by shear has been briefly mentioned previously by Goldstein and Reichmann.[18]

The theoretical calculations show that the rupturing stress applied by the viscous forces is maximal in the center of the chain and increases as the square of the length of the molecules. The molecules should thus break roughly in half when the critical shear is applied, and a very long molecule should be halved repeatedly until a fairly uniform population of chains stable to the shear gradient is obtained.

No demonstration of heterogeneity in the undegraded phage DNA has been achieved, but the sharp skew peak may indicate a complexity which could be resolved at lower concentrations. Unfortunately the optical limitations at present preclude the employment of solutions much below 0.0005 per cent concentration for accurate analyses. It is known that self-sharpening effects persist in normal DNA preparations down to 0.01 per cent, and it may not be unexpected that in the presence of much longer chains the critical concentration should be much lower. However, it is also possible that the DNA population is essentially homogeneous (apart from aggregates). The Doty formula,[6] if it were applicable, would indicate a molecular weight about 1×10^8 (roughly the DNA content of a phage particle) for a 56 S molecule. If the DNA is normally present as one macromolecule, the autoradiographic experiments must be explained by degradation of this molecule, presumably during the manipulation of the emulsion. This appears unlikely since the application of controlled shear gradients did not suffice to produce a bimodal distribution. However, it is obvious that for an answer to the present difficulties some correlation of molecular weight and sedimentation coefficient is needed for the phage DNA.

Nothing in the experiments reported in this paper has shown that the DNA sedimenting in these sharp peaks is protein-free, although the fact that the material in some experiments has been banded at a specific gravity of 1.7 before study implies that any protein present must be in small proportion. Thus it could be postulated that protein bridges exist along the DNA macromolecule uniting DNA sub-units. It can also be postulated that the shear scission occurs at these or other weak points. If they are present, such bridges must be resistant to chymotrypsin. However, until there is some definite evidence, it seems most reasonable to assume that the hydrodynamic shear is transecting the DNA double helix.

It should perhaps be pointed out that the phage particle itself would suffer no embarrassment from the shear sensitivity of its DNA during the process of injecting these molecules into the bacterium, since the diameter of its tail is too small for the DNA to lie across the streamlines, even if there were a solvent present to impart a viscous drag.

The observed lability of the phage DNA poses the problem of how much the DNAs from other tissues might be degraded in the course of preparation. The relative uniformity of observed molecular weights in sperm and thymus DNA preparations, for example, is no proof that these molecules have not been degraded, since a fairly uniform distribution would be expected from the shearing action. The

vigorous stirring, blending, or filtration to which most preparations of DNA are subjected would certainly degrade phage DNA. If DNA *in vivo* is much longer than is usually recognized, some of the present problems of correlating DNA structure and function would be simplified. Beiser, Pahl, Rosenkranz, and Bendich[19] found that specific pneumococcal transforming activities were chromatographically complex. Assume, for simplicity, that each activity relates to the presence of one enzyme. If the DNA molecule is very long and is ruptured during isolation or deproteinization to give chains of 8×10^6, average molecular weight (of which 1×10^6 would be needed to code for a protein of 50,000 molecular weight[20]), the activity might be found on molecules of variable length and composition. Little loss of activity need be envisaged since only one break in eight would interrupt the critical sequence; if the sequence lies close to the end of the gross macromolecule, the sequence would rarely be ruptured.

Furthermore, at present most speculations on chromosome structure assume that the DNA is of relatively uniform molecular weight (about $4–8 \times 10^6$). The evidence presented in this paper shows that the experimental grounds for this assumption are questionable. It is obvious that some of the earlier physical work on DNA must be repeated with suitable precautions to avoid the scission of any very long molecule which may be present.

Summary.—(1) Turbulence and high shear gradients have been shown to cause a decrease in the sedimentation coefficients of T_2 and T_4 bacteriophage deoxyribonucleic acid molecules in solution. (2) In sedimentation velocity experiments, carefully treated DNA solutions have shown a sharp peak, skew on the high $S_{20,w}$ side, with a maximum about 60 S.

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† Throughout this paper the word "degradation" is used to denote a reduction in molecular weight—presumably by transverse scission of the DNA double helix.

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THE PHOTOCHEMICAL OXIDATION OF DPNH WITH RIBOFLAVIN PHOSPHATE

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Recently this laboratory has reported studies on the non-enzymatic catalysis of oxidation by flavin coenzymes of various nitrogen compounds in light.[1,2] In an extension of these studies to other coenzymes, we have found that reduced diphosphopyridine nucleotide (DPNH) can also be efficiently oxidized photochemically by

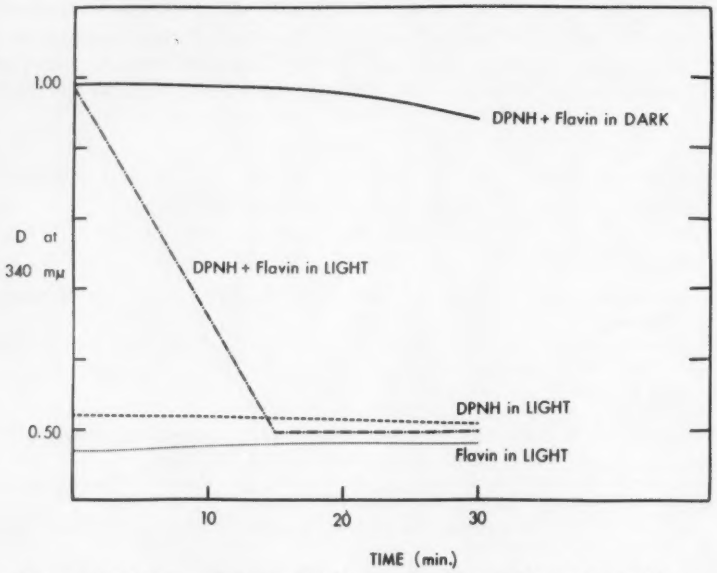

FIG. 1.—Oxidation of DPNH by riboflavin phosphate in light under aerobic conditions. Quartz cuvettes contained 0.27 μmole of DPNH (Sigma Chemical Co.) and/or 0.25 μmole of riboflavin phosphate (Sigma Chemical Co.) in 3.0 ml of 0.075 *M* potassium phosphate buffer (0.0001 *M* in Mg^{++}), pH 7.8. For illumination, cuvettes were placed 2 cm from 36-in. General Electric "black light" fluorescent lamp, and "dark" controls covered with black paper. Optical densities at 340 mμ measured with Beckman DU spectrophotometer.

riboflavin phosphate in the absence of apoenzymes or any other organic compounds. Typical results are presented in Figure 1. Thus, it will be seen that when a solution

of DPNH plus riboflavin phosphate is illuminated aerobically, the DPNH rapidly disappears as evidenced by the loss of the characteristic 340 mμ absorption. On the other hand, the level of DPNH is not significantly decreased when the same mixture is kept dark or when the DPNH is illuminated in the absence of the flavin. The 340 mμ absorption of flavin itself remains constant in these experiments.

Following exposure of the DPNH-riboflavin phosphate mixture to light and the disappearance of the 340 mμ absorption, treatment of the solution with dithionite[3] regenerates the DPNH quantitatively, as shown in Table 1. Further illumination

TABLE 1

PHOTOOXIDATION OF DPNH WITH RIBOFLAVIN PHOSPHATE AND QUANTITATIVE REGENERATION OF THE DPNH BY DITHIONITE TREATMENT

	D 340 mμ
DPNH + Flavin:	
(a) Before illumination	0.383
(b) After illumination	0.007
(c) Solution (b) after dithionite treatment	0.385
(d) Solution (c) after second illumination	0.007

Rectangular quartz cuvettes contained 0.27 μmole DPNH and/or 0.25 μmole riboflavin phosphate in a total volume of 3.0 ml of 0.075 *M* potassium phosphate buffer (0.0001 *M* in Mg^{++}), pH 7.8. Reagent "blank" for spectrophotometric measurements was the solution containing only the flavin. 0.7 ml. of 0.2 per cent dithionite (in 1% $NaHCO_3$) employed for DPNH recovery. Conditions for illumination same as given in Fig. 1.

of this same solution leads again to oxidation of the DPNH. It can be concluded, therefore, that the loss of two electrons is the major, if not the only, chemical change incurred by the DPNH molecule when illuminated in the presence of the flavin.

It now became pertinent to determine whether the riboflavin phosphate is reduced to its leuco form concurrently with the photoxidation of the DPNH. Under the aerobic conditions of the experiments described in Figure 1, it is not possible to isolate the reduced form of the flavin because of its rapid oxidation by molecular oxygen.[2] Thus, as seen in Figure 2, the oxidized form of the riboflavin phosphate is recovered almost quantitatively from the reaction mixture when the oxidation of the DPNH is complete. Consequently, the photooxidation of DPNH with the flavin was carried out in the absence of oxygen by flushing the system with nitrogen prior to illumination. Upon exposure to light this anaerobic DPNH-flavin mixture immediately blanched, as evidenced by the loss of both the yellow color and the fluorescence. Spectroscopic analysis also showed that the 445 mμ peak of the flavin had disappeared. When oxygen was again admitted to the system, the yellow color and fluorescence characteristic of the oxidized flavin reappeared immediately. No change in the fluorescence and color of the control solution containing only the flavin could be observed during this experiment. These results demonstrated unequivocally that the riboflavin phosphate is the electron acceptor in the photooxidation of the DPNH and also that the DPNH is the predominant, and perhaps only, source of electrons in the system. The stoichiometry of the electron transfer can therefore be described by the following equation:

$$\text{DPNH} + \text{H}^+ + \text{Flavin} \xrightarrow{h\nu} \overset{+}{\text{DPN}} + \text{Flavin}\cdot 2\text{H}$$

From our previous findings[2] it may be presumed that the subsequent reoxidation of the leuco form of the flavin with molecular oxygen yields hydrogen peroxide.

Our earlier studies showed that the riboflavin phosphate can serve a dual function

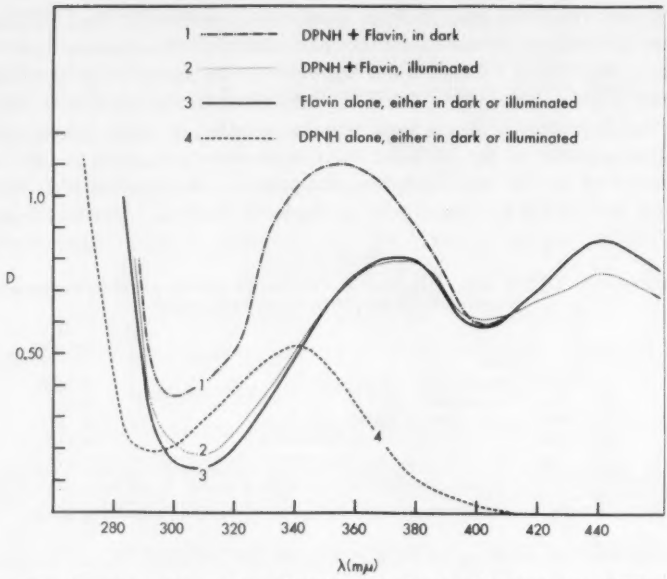

FIG. 2.—Absorption spectra of DPNH, riboflavin phosphate, and DPNH plus riboflavin phosphate before and after illumination under aerobic conditions. Absorption spectra of solutions employed in experiment of Figure 1 were measured in Beckman DU spectrophotometer.

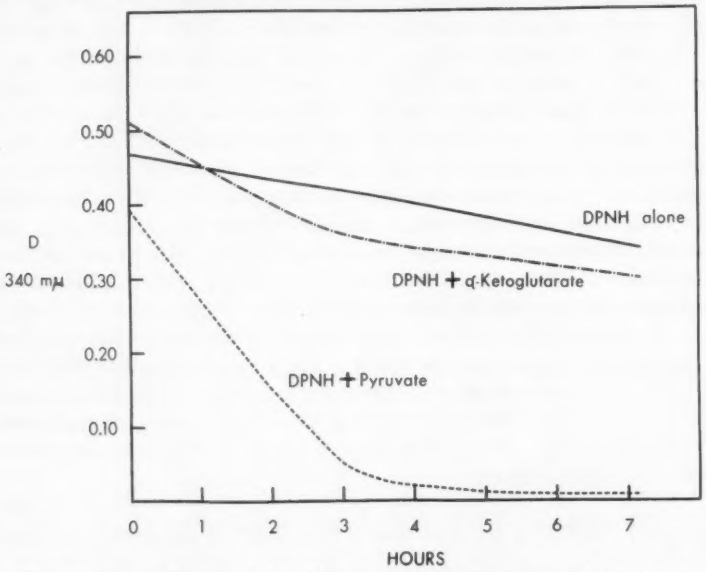

FIG. 3.—Photooxidation of DPNH in presence of pyruvate or α-ketoglutarate. 0.27 μmole of DPNH and 30 μmoles of sodium pyruvate or sodium α-ketoglutarate (Nutritional Biochem. Corp.) dissolved in 3.6 ml of 0.075 M potassium phosphate (0.0001 M in Mg^{++}), pH 7.8, and illuminated as described in Figure 1.

in photooxidations, acting both as the electron acceptor and, in its photoactivated state, as the source of the activation energy for the dehydrogenation of aliphatic nitrogenous "substrates." There was no indication that the latter compounds were photoactivated during this process. DPNH, on the other hand, can be photoactivated, as evidenced by its ability to fluoresce, and it is not unlikely that *both* the DPNH and the riboflavin phosphate are therefore activated in the electron transfer reaction described above. Indeed, the most direct evidence that DPNH itself becomes reactive in light is provided in other experiments, summarized in Figure 3, in which we have found that "black light" illumination of a buffered mixture of DPNH and pyruvate leads to rapid oxidation of the coenzyme. Furthermore, it should be noted that this photoactivated removal of H from the DPNH in the presence of the keto acid exhibits considerable structural specificity, as demonstrated by the fact that the rate of reaction with α-ketoglutarate is appreciably lower than with pyruvate.

The photochemical oxidation of DPNH by riboflavin phosphate reported here recalls the observations by Singer and Kearney[4] on the non-enzymatic reduction of ferricytochrome C by DPNH, mediated by various flavins, and the more recent studies of Suelter and Metzler[5] on the non-enzymatic oxidation of DPNH by riboflavin derivatives. Although no mention of any effect of light was made in either report, it is not unlikely that these reactions could also be stimulated by light. The photochemical oxidation of DPNH by flavin demonstrated by our experiments also suggests a possible mechanism for the phenomenon of "photo-reactivation" described by Dulbecco.[6] He has found that when bacteria are damaged by ultraviolet light, they can be reactivated by light of longer wavelengths and that the action spectrum of this reactivation has a peak at about 3,500 Å. The fluorescent light source employed in our studies has a maximum emission of this same wavelength.

Of the non-chemical forms of energy, light can be most efficiently transformed into chemical energy without profoundly altering the receptor system or its surroundings. Consequently, the capture of light energy and its translation into chemical work may have been an event of considerable importance in the evolution of organic and biochemical reactions. Such absorbed light could have provided the energy of activation required for the catabolism of primordial organic compounds as well as the energy for driving anabolic reactions. In the former case both the compounds produced and the energy liberated could have been used, under favorable conditions, for the synthesis of larger and more complex molecules. If light played such a role, it is probable that compounds which possessed the ability to absorb light and function as catalysts in primitive organized systems may have continued to function catalytically in biochemical reactions even after the origin of proteins.

Both riboflavin and DPNH, ubiquitous in their occurrence, are prime examples of such catalytically active molecules and the significance of their photosensitivity must be considered in any comprehensive hypotheses of chemical evolution. As recently reported from this laboratory,[2] a number of simple nitrogenous compounds are formaldehydogenic when exposed to light and riboflavin phosphate. Relevant to this finding, it was pointed out that not only have formaldehyde and its products been underscored in theories of the origin of life,[7] but that the same compounds which were found to be formaldehydogenic have also been proposed as primordial

substances.[8] Our current demonstration of the photoreactivity of DPNH, still another vitamin derivative, lends an even wider perspective to the plausible implications of such compounds in evolutionary processes. Being the most abundant and universal of the contemporary respiratory coenzymes, the pyridine nucleotides also lie closest to the metabolic "substrate levels" and are both the initial electron acceptors in catabolic processes as well as the final hydrogen donors in synthetic reactions. The simple experiments described here provide a model system for demonstrating how the interplay of light and a reduced coenzyme can lead to the transfer of electrons either in the "substrate direction" or toward other oxidants, including molecular oxygen. In this respect, the action of light could modify a delicate balance between processes of synthesis and degradation both in primordial and contemporary life. Further experiments on the photodynamic reactions of vitamins, nucleotides, and other heterocyclic nitrogenous bases are now in progress.

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ELEMENTS OF A GENERAL THEORY OF ENZYMATIC HYDROLYSIS

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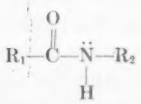
Communicated by Albert Szent-Györgyi, September 14, 1959

The following propositions seem to us to form a suitable basis for a general or, at least, a fairly general theory of the mechanism of enzymatic hydrolysis:

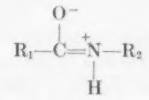
First proposition. In practically all (in fact, in all but one) fundamental types of biochemical substrates undergoing enzymatic hydrolysis the bond which is hydrolyzed carries formal positive charges on its two extremities. We shall call it a "dipositive bond."

This notion of a dipositive bond may need an explanation. Let us consider, as an example, the peptide bond:



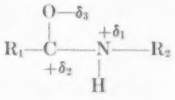
This bond is formed of a basic skeleton of single localized (σ) links, upon which there is superposed a *system of mobile or π electrons.* This system is composed in this case of four electrons: two electrons of the double bond of the C=O link and two electrons of the lone pair of the nitrogen atom. These four electrons constitute a resonating system and, as a general rule for cases where there is a lone pair containing

heteroatom adjacent to a double bond, the resonance will result in a partial transfer of the lone pair from this heteroatom to the terminal atom of the double bond. This may be considered as resulting from the contribution of a formula of the type



to the real structure of the peptide linkage. As a result of this contribution the nitrogen atom will bear a *formal positive charge* which means that, instead of carrying the two electrons of its lone pair, it will carry only a part of them.

Moreover, owing to the difference in electronegativity between carbon and oxygen, the carbon-oxygen double bond may be considered as being a resonance hybrid of the covalent C=O structure and the ionic $C^+—O^-$ structure, so that the carbon atom of the peptide link must also bear a formal positive charge, which means that it will carry less than the one π electron which it contributes to the π electron pool. The oxygen will, on the whole, carry more than the one π electron which it contributes to the π-electron pool and will thus bear a formal negative charge. All these formal charges are measured in fractions of electrons and if we denote these fractions by the general symbol δ the distribution of the formal charges on the peptide link may be represented by:



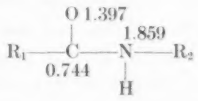
with obviously

$$\delta_3 = \delta_1 + \delta_2$$

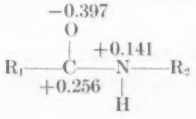
and

$$(1 + \delta_3) + (1 - \delta_2) + (2 - \delta_1) = 4$$

This general result is independent of the precise values of the parameters adopted in the effective calculations. As an example, and only in order to fix the idea, we reproduce here, nevertheless, the results of calculations, carried out, in fact, by the molecular orbital method,[1] for the peptide link with a certain set of parameters for the different coulomb and exchange integrals. The distribution of the four π electrons of the system is the following:



from which the following distribution of the formal charges is deduced:



The denomination of "dipositive bond" which we apply to the C=N bond of the peptide linkage refers to this deficiency of π electrons on both atoms which constitute this bond. This dipositive character exists before any external perturbation, including attack by an enzyme.

Besides this polarization of the π electrons there is also a polarization of the underlying σ electrons, due to the difference in electronegativities of the bonded atoms. This polarization of the σ bonds may sometimes be in a direction opposite to the polarization of the π electrons cloud. Thus, while the σ bond of the C—O link is polarized in the same direction as its π electron bond (toward the oxygen) the C—N σ bond is probably polarized toward the nitrogen atom. Nevertheless, we do not need generally to take this polarization of the σ bonds into consideration because the chemical (or biochemical) reactivity of systems possessing π electrons is generally due essentially to these π electrons. These electrons, being more loosely bound than the σ ones, take part more easily in chemical reactions.

We may now come back to our first proposition, namely, that in all fundamental biochemical substrates but one, the bonds which undergo enzymatic hydrolysis are of this dipositive character. Effectively these fundamental biochemical substrates include, beside the peptide link:

(1) *The ester link*, which may be a carboxylic ester $R_1—\overset{\overset{O}{\|}}{C}—O—R_2$ a phenolic ester Ph—O—R, a phosphoric ester $R—O—\overset{\overset{\|}{}}{\underset{\diagdown}{P}}—$, a sulfuric ester $R—O—\overset{\overset{\|}{}}{S}—$ etc.

(2) *The acid anhydride link*, in particular the $—\overset{\overset{\|}{}}{\underset{\diagdown}{P}}—O—\overset{\overset{\|}{}}{\underset{\diagdown}{P}}—$ link in the energy rich phosphates.

(3) *The amines*, e.g., the $—\overset{\diagdown}{\underset{\diagup}{C}}—NH_2$ link in adenine or quanine.

(4) *The guanidines*, e.g., the $—NH—\overset{\diagup}{\underset{\diagdown}{C}}—$ link in arginine or creatine.

(5) *The glycosidic linkage* of the polysaccharides.

(6) *The N-glycosidic linkage* of ribosides of purines, pyrimidines, etc.

Now, all these links, with the only exception of the glycosidic linkage of the polysaccharides, are dipositive bonds in the sense defined previously. The different character of the glycosidic linkage of the polysaccharides results from the fact that this linkage, and this linkage only, does not involve a system of π electrons but only a chain of single bonds. So that in this particular case it is only the polarization of the σ skeleton which plays a role. Under these conditions, the inductive effect of the central oxygen atom creates a certain complementary concentration of electronic charge on it (makes it slightly negative), while the carbons joined to it are electron deficient (partially positive).

The dipositivity of the hydrolyzable bonds may be demonstrated by general considerations similar to those developed for the peptide linkage. The general result is independent of the precise parameters adopted in calculations. Nevertheless, for

simplicity, we shall reproduce here one or two representative examples for each case (Fig. 1):

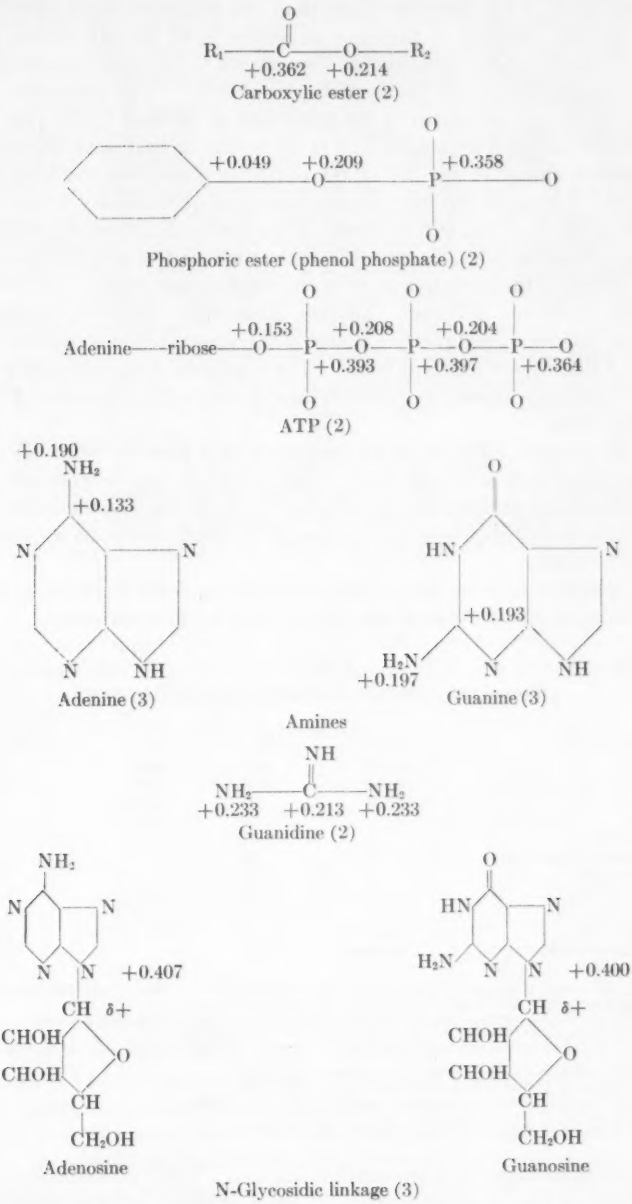


FIG. 1.—The dipositive character of hydrolyzable bonds.

Figure 1 may need two comments.

(a) It may be observed that in some cases, e.g., in the phosphoric (or sulphuric etc.) esters and in ATP, there are more than two adjacent atoms carrying formal positive charges. Instead of having a dipositive bond we have there, in fact, a polypositive chain. This situation has been shown to constitute a general and important characteristic of the energy rich phosphates.[2, 4] In connection with our present study it may be related to the possibility of different hydrolytic splittings.

(b) In case of the *N*-glycosidic link of adenosine or guanosine the value of the formal positive charge of the N9 of the purine is given, but the formal positive charge of the C atom of the sugar is only indicated qualitatively by the symbol δ. This is due to the fact that the positivity of this carbon is difficult to evaluate: it is not due to π electron delocalization (this carbon is a part of an unconjugated sugar ring) but simply to the inductive effect of the neighboring oxygen. Whatever it be, this carbon is electron deficient. Another interesting remark in connection with this point is that the formal positive charge of this sugar carbon is probably the same in a series of ribosides of different bases. The positivity of the nitrogen atom of the *N*-glycosidic linkage of these ribosides will thus be even a direct measure of the dipositivity of this bond.

Second proposition. The enzymatic hydrolysis takes place the easier the greater the electron deficiency of the susceptible link. This deficiency may be increased by the increase of the formal positive charge either of one of its constituent atoms (in which case this atom is probably in some way one of the reactive centers for the reaction) or of its two atoms.

This proposition may be illustrated by comparing some available experimental results of calculations of the electronic distribution in these substrates.

TABLE 1

HYDROLYSIS OF THE N- GLYCOSIDIC LINK OF RIBOSIDES

Compound	The Rate of Hydrolysis Ref.[a]	Ref.[b]	Ref.[c]	The Formal Charge of the N Atom of the Glycosidic Link
Adenosine	0.89	103	490	+0.407[d]
Inosine	0.95	100	470	+0.419[d]
Guanosine	0.40	90	600	+0.406[d]
Xanthosine		<1	150	+0.414[d]
Purine riboside		47		+0.408[d]
2,6-diaminopurine riboside	0.37	76		+0.399[e]
Uric acid riboside		<1		+0.277[f]
Cytidine	0	54	100	+0.361[g]
Uridine	0	7	0	+0.311[g]
Thymidine	0	4		+0.307[g]
Nicotinamide riboside	≃inosine	150		+0.422[h]

[a] Heppel, L. A., and R. J. Hilmoe, "Hydrolysis by enzymes from yeast, *J. Biol. Chem.*, **198**, 683 (1952). [Listed is the quantity of reducing sugar (in μM) formed after 5 hours incubation at 37°.]

[b] Takagi, Y., and B. L. Horecker, "Hydrolysis in L. Delbrueckii, *J. Biol. Chem.*, **225**, 77 (1957). [Comparative rates of hydrolysis in which the rate of cleavage of inosine is represented by 100].

[c] Tarr, H. L. A., "Fish muscle riboside hydrolases, *Biochem. J.*, **59**, 386 (1953). [Ribose formed (μg/ml) after 3 hours at 37°, pH 5.5].

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[h] Pullman, B., and A. Pullman, these PROCEEDINGS, **45**, 136 (1959).

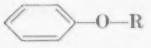
One group of such results is presented in Table 1. This table contains the experimental results of the rate of hydrolysis of a series of ribosides (*N*-glycosidic linkage) and the results of calculations of the positive charge on the involved nitrogen atoms

(the positive charge on the C atom of the glycosidic ring being, according to a previous remark, practically constant in all these ribosides, the charge on the N atom is a direct measure of the relative electron deficiency of the hydrolyzed bonds.)

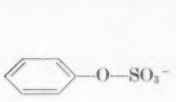
It may be observed that in spite of some discrepancies in the experimental results of the different authors quoted, the ribosides of purines appear, in general, as being more easily hydrolyzed than the ribosides of pyrimidines. Of the former, the riboside of uric acid appears to be exceptionally stable; of the latter, cytidine seems to be more easily hydrolyzed than uridine and thymidine. Finally, it may be remarked that the riboside of nicotinamide is particularly easily hydrolyzed.

The value of the formal positive charge on the nitrogen atom of the hydrolyzed bond accounts very satisfactorily for these general results: it is greater, on the average, in the purine ribosides than in the pyrimidine ribosides, with the exception of the riboside of uric acid in which it is particularly small; it is greater in cytidine than in uridine or in thymidine; and it is the greatest among all the studied compounds in the riboside of nicotinamide. A more detailed examination of the correlation especially inside the series of the purine ribosides shows some discrepancies, which need a further investigation. As a matter of fact, such discrepancies exist also between the different experimental results and are certainly related to the *specificity* of each of these enzymatic reactions.

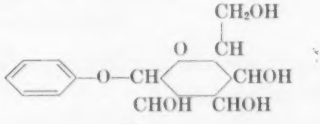
Another group of examples which illustrate the proposition that the enzymatic hydrolysis takes place the easier the greater the electron deficiency of the bond to be hydrolyzed, may be found in the field of *the influence of substituents on the ease of enzymatic hydrolysis of phenyl esters.* These are compounds to the general formula:



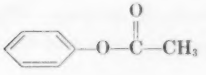
Among the compounds effectively studied in this respect we may quote the derivatives of phenylsulfate (I),[5] phenyl-β-D-glycoside (II)[6] and phenylacetate (III).[7] The case of derivatives of benzoylcholine (IV)[8] is strictly related.



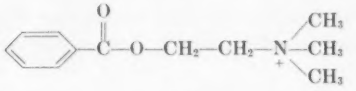
I



II



III

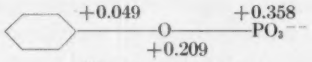


IV

Now, in spite of important differences in the detailed mechanism of their enzymatic hydrolysis (which we shall discuss in a forthcoming publication), *all these substrates have in common the fact that the substitution of electron withdrawing substituents on the phenyl ring (especially in the ortho and para positions) facilitates the hydrolysis while a similar substitution of electron repelling substituents decreases the rate of hydrolysis.* This influence of substituents may obviously be related to the effect of these groups on the charge concentrations on the splitted bonds and it has been so related by the authors of these different experiments. Nevertheless, in connection

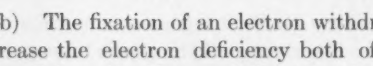
with the different interpretations suggested, the following observations may be particularly useful:

(a) In all these different esters we have at the extra ring chain at least three adjacent atoms carrying a formal positive charge. We have thus at least two dipositive bonds there. The situation may be illustrated by available calculations which, although they do not concern the esters quoted in this paper, are, nevertheless, representative of all these types of compounds. The available calculations concern the phenylphosphate.[2]

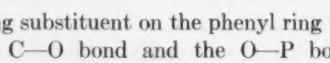


Phenylphosphate

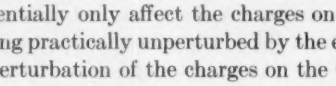
(b) The fixation of an electron withdrawing substituent on the phenyl ring will increase the electron deficiency both of the C—O bond and the O—P bond. Nevertheless, it will essentially only affect the charges on the C and O atoms, the charge on the P atom being practically unperturbed by the effect of a substitution on the phenyl ring. The perturbation of the charges on the C and O atoms will consist, for both these atoms, in an increase of their formal positive charges, particularly when the substitution occurs at the *ortho* or *para* positions. The phenomenon is illustrated in the following diagrams which show the distribution of the formal positive charges on the involved positions in the three isomeric nitrophenylphosphates.



Para nitrophenylphosphate



Ortho nitrophenylphosphate

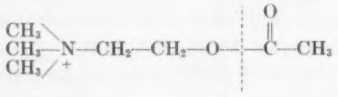


Meta nitrophenylphosphate

In benzoylcholine the perturbation will affect essentially, of course, the carbon atom of the carboxyl group.

This last remark leads us to quote one more example, which illustrates in a particularly striking way the predominant importance of the degree of the electron deficiency of the susceptible bond for the ease of its enzymatic hydrolysis. The example is concerned with the *action of cholinesterase on acetylcholine and related compounds.*[9]

Acetylcholine has a dipositive acyl ester bond indicated at the formula and there are different experimental evidences



that the enzymatic hydrolysis occurs effectively on this bond.

Moreover:

(a) The hydrolysis is easier in the halogenoacetate derivatives. The halogens

increase, of course, the electron deficiency of the acyl-ester bond, essentially by increasing the positivity of the C carbon of the carboxyl bond.

(b) On the contrary, the replacement of CH_3 by NH_2 or OH prevents any hydrolysis from occurring. These substituents which are electron donors decrease appreciably the electron deficiency of the acyl-ester bond.

(c) The corresponding thioester in which an S atom has taken the place of the ethereal O atom hydrolyses as easily, or even easier, than acetylcholine. The lone pair on the sulfur atom being more loosely bound than that of an oxygen, the sulfur atom has a greater formal positive charge than the corresponding oxygen. The influence of this replacement on the positive charge of the adjacent C atom is more difficult to ascertain. Calculations are in progress to settle this point.

(d) On the contrary, the formation of a thioester, by replacing the oxygen atom of the C=O bond by a sulfur atom, results in a total inhibition of hydrolysis. Of course, this replacement, because of the much smaller electronegativity of sulfur with respect to that of oxygen, decreases to a very large extent the ionic character of the C=O bond and consequently practically abolishes the formal positive charge of the C atom.

These results represent thus a very clear demonstration of the importance of the concentration of the formal positive charges on the susceptible bond for the occurrence and ease of this enzymatic hydrolysis. They have been considered as such by F. Bergmann.[9]

Third proposition. When several similar bonds are present in a molecule and when steric hindrance or specificity may probably be disregarded, the enzymatic hydrolysis occurs at the most dipositive of these bonds.

A most striking example of this proposition is offered by allantoin, the product of oxidative breakdown of uric acid. This molecule possesses five similar bonds of the peptide type. The bond which first undergoes enzymatic hydrolysis[10] is indicated by the dotted line on Figure 2 which also reproduces the distribution of the formal positive charges in this compound.[11]

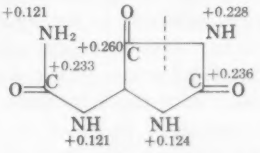


FIG. 2.—Formal positive charges in allantoin.

It can be seen that the hydrolyzed bond is by far the most dipositive one, the formal positive charges on both its C and N atoms being greater than those of the corresponding atoms of the other similar bonds of the molecule.

Another similar example is barbituric acid (Fig. 3) whose enzymatic hydrolysis, to urea and malonic acid, also occurs along the most dipositive bonds.[12]

Conclusion.—In view of the preceding discussion it seems thus obvious that *the electronic deficiency of the bond which will undergo enzymatic hydrolysis is an important factor in determining the occurrence and the ease of this reaction.* Nevertheless, this deficiency is a feature characteristic of the substrate and the question may be raised of what the role is of the enzyme in this type of reaction. The answer would be

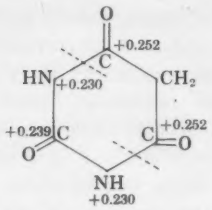


FIG. 3.—Formal positive charges in barbituric acid.

that this role may probably be multiple and that the catalytic action of the enzyme may occur, and most certainly does so, in many different ways in different specific reactions. We shall present, in a forthcoming paper, a detailed discussion of the mechanism of a series of fundamental enzymatic hydrolytic reactions and, consequently, we shall indicate here only schematically the principal possibilities.

It seems highly probable that the catalytic action of the enzyme will be determined, more or less following the case, by the pre-existing electronic situation. The simplest mechanism by which the positivity of the susceptible link may be associated with the action of the enzyme would consist in *a direct attack of a nucleophilic center of the enzyme on one or the other of the positive centers of the hydrolyzable bond.* A formation of such a direct complex with the reactive center may favor the fission of the link and the subsequent hydrolysis. A mechanism of this type is probably involved in the action of cholinesterase on acetylcholine and its derivatives, which we have previously discussed.[9] It may also be involved in the mechanism of action of a number of esterases with a reactive serine residue and which may be inhibited by organophosphorus compounds,[13] etc.

Another mechanism relating the action of the enzyme to the pre-existing electron deficiency may consist in a tendency of the enzyme to *increase this pre-existing deficiency* by increasing the formal positive charges either on both atoms constituting the reactive bond or on one of them. This may be obtained on behalf of the enzyme by forming appropriate "bonds" with the substrate which will tend to modify the electronic distribution on the substrate in the desired direction. This binding may occur in positions more or less close to the susceptible bond and may result in favoring either the fission of the bond or the attack of one of its extremities by nucleophilic agents. In connection with this hypothesis a special mention must be made of a theory of enzymatic hydrolysis, proposed by Ronwin[14] which was based on the idea that the role of the enzyme consisted in "*creating*" a dipositive charge situation at the susceptible link which leads to a ruptured or distorted bond with subsequent hydrolysis." This author had thus to imagine the mechanisms by which such a dipositive situation may be induced. In fact, as we have seen, it is already there, before any action on behalf of the enzyme.

On the other hand, it must also be borne in mind that this electron deficiency although apparently frequently essential for the mechanism of the hydrolysis need not be directly involved in the interaction of the enzyme with the substrate. This interaction may occur far away from the susceptible bond and have the essential effect of helping in some way (orientation effects, strain, Van der Waals binding) to lower the activation energy for the hydrolysis itself.

These are only a few of the possible ways in which enzyme catalysis may be re-

lated to the positivity of the susceptible bond in hydrolytic reactions. It must be borne in mind that very different mechanisms may be involved even in apparently related reactions. Thus, in spite of the apparently similar effect of substitutents on the yield of the reaction it seems highly probable that the detailed mechanism of the hydrolysis is quite different in the different types of phenylesters which we have quoted. The problem, as stated, will be discussed in more details separately.

Finally, we should like to stress that in no way do we want to suggest that the electron deficient character of the susceptible bond is the only factor which determines the occurrence and the ease of enzymatic hydrolysis. It is obvious that other factors which include, for example, steric specificity, shape and size, strain and polarization, electrostatic interactions and long-range forces, are as important as the factor that we have discussed here. Most probably they are frequently complementary to each other and it must be remembered that even in a case such as that of the cholinesterase action on acetylcholine and its derivatives, in which it seems very probable that the mechanism of the reaction consists essentially in a direct interaction between a nucleophilic group of the enzyme and a positive center of the bond, there is ample evidence that the binding with the enzyme involves also a second site on the molecule, probably the quaternary nitrogen.[15] Such a complementary binding, which is probably frequent, may, of course, play a very important role in the over-all mechanism. Nevertheless, what seems highly probable from the present study is *that the electron deficiency of the susceptible bond is one of the essential electronic factors, if not the essential electronic factor*, which determines the mechanism of the reaction. It may also be mentioned that the case of the polysaccharides, although to some extent particular in our scheme (because their hydrolyzable bond is not a dipositive one) probably does not present any fundamental difference with respect to the other groups of hydrolyzable substrates. The susceptible bond in these substances contain, because of the polarization of the σ bonds, electron deficient carbon atoms and this deficiency may be an essential element for the reaction.

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[1] For a general description of the method, see Pullman, B., and A. Pullman, *Les Théories Électroniques de la Chimie Organique* (Paris: Masson Ed., 1952).

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[9] Bergmann, F., in *Advances in Catalysis*, **10**, 130 (1958).

[10] Di Carlo, F. J., A. S. Schultz, and A. M. Ken, *Arch. Biochem. Biophys.*, **43**, 468 (1953).

[11] Unpublished calculations by the authors.

[12] Pullman, A., and B. Pullman, *Bull. Soc. Chim. France*, 594 (1959).

[13] See, e.g., Porter, G. R., H. N. Rydon, and J. A. Schofield, *Nature*, **182**, 927 (1958); Rydon, H. N., *Nature*, **182**, 928 (1958).

[14] Ronwin, E., *Enzymologia*, **16**, 81, 179 (1953–1954), *J. Amer. Chem. Soc.*, **75**, 4026 (1953); see also Ferguson, L. N., *Enzymologia*, **17**, 95 (1954); **18**, 273 (1957).

[15] See, e.g., Wilson, I. B., in *The Mechanism of Enzyme Action* (Baltimore: Johns Hopkins Press, 1954).

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*A SULFOLIPID IN PLANTS**†*

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A sulfolipid-S^{35} was isolated from photosynthetic microorganisms and higher plants after culture in media containing radiosulfate. Alcohol extracts of *Chlorella*, *Scenedesmus*, *Rhodospirillum rubrum*,[1] barley, clover, New Zealand spinach (*Tetragonia expansa*) chloroplasts, chive, and green sections of coleus were chromatographed on paper and found to contain varying amounts of a radioactive compound with high R_f values (Fig. 1). Its concentration in *Chlorella* was $4 \cdot 10^{-3}$ *M*, which exceeds those of the major plant phosphatides. The radiochromatographic method has revealed its chemical composition and aspects of its molecular structure.

FIG. 1.—Radiogram of alcohol extract of *Chlorella*-S^{35}. Chromatograms are developed from the origin at the lower left with phenol-water (1) to the right and with butanol-propionic acid-water (2) upwards.

Materials and Methods.—Isolation of sulfolipid-S^{35}: A suspension of approximately 100 mg of *C. pyrenoidosa* or of *Scenedesmus* D_3 in 30 ml of low-sulfur (*ca.* 10^{-5} *M* total sulfates) culture medium containing 1 mc carrier-free sulfate-S^{35} in a glass cylinder was perfused with 5 per cent carbon dioxide-enriched air and illuminated with a 20-watt fluorescent lamp. During one week the cells increased fivefold. They were centrifuged from the medium and washed three times with water. The cells were extracted twice with 5-ml portions of hot absolute ethanol and twice with hot 25 per cent chloroform in ethanol. The extract of 20-mg cells was chromatographed two-dimensionally on Whatman No. 1 paper in phenol-water (PW) and

butanol-propionic acid-water (BPAW)[2] solvents. The sulfolipid had an R_f of 0.8 in PW and 0.7 in BPAW (Fig. 1). This allowed separation from the slower moving phosphatidyl inositol in subsequent experiments. The sulfolipid contained 90 per cent of the S^{35} activity of the chloroform-ethanol extract. It was eluted from chromatograms with 25 per cent chloroform in ethanol or with pyridine.

In analogous experiments the leaves of higher plants were labeled during 10–20 hours' illumination while their petioles were immersed in radiosulfate solutions. Extractions and isolation of the lipid gave in each case a product with identical R_f values and chemical properties.

Isolation of sulfolipid-C^{14}: A suspension of 100 mg of *Chlorella* in 30 ml of culture medium in a glass cylinder was connected in a closed system to a gas-generating flask and a valveless circulating pump.[3] $C^{14}O_2$ was introduced by addition of 0.5-mc portions of barium carbonate-C^{14} to dilute lactic acid at two-day intervals. After eight days the weight of the algae was 400 mg. They were separated from the growth medium and extracted with 10 ml of boiling 80 per cent ethanol. Further extracts with 10-ml portions of hot 95 per cent ethanol and 10 ml of hot 25 per cent ethanol-chloroform were combined and concentrated *in vacuo*. Chromatography in PW and BPAW effected complete separation of the sulfolipid-C^{14} from the other labeled components. Phosphatidyl inositol occupied an adjacent area and was recognized by conversion to fatty acids and glycerophosphoryl inositol in the anticipated C^{14} activity ratio. No other phosphatides or sulfolipids occupy this region of the chromatograms of *Chlorella* extracts.

The identity of the sulfolipid-C^{14} with the sulfolipid-S^{35} was confirmed by conversion by acid hydrolysis and by deacylation to the readily recognizable sugar-6-sulfonate and glyceryl glycoside-6-sulfonate. In all cases the rates of hydrolysis of the C^{14}- and S^{35}-labeled lipids were identical.

Experiments and Results.—Structural aspects of the sulfolipid were deduced from the chromatographic properties and chemical reactivities of its components. Their chemical composition was revealed by the relative C^{14} radioactivity in each moiety derived from uniformly labeled sulfolipid-C^{14}.

Acid hydrolysis of sulfolipid-S^{35}: A solution of 10,000 cpm of sulfolipid-S^{35} in 0.10 ml 50 per cent ethanol was added to 0.05 ml of 6 *N* hydrochloric acid and heated at 100° for 30 minutes. Chromatography of the hydrolysate revealed but one sulfur-containing product with R_f values of 0.05 in PW and BPAW. Rapid paper electrophoretic migration with lactic acid-hydrochloric acid at pH 1 and with boric acid-sodium hydroxide buffer at pH 9 indicated its strong anionic nature. No inorganic sulfate was produced by heating at 100° for ten hours in normal hydrochloric acid or for two hours in 50 per cent nitric acid.

Base-catalyzed deacylation[4,5] of sulfolipid: To 10,000 cpm of sulfolipid-S^{35} in 0.05 ml toluene-methanol was added 0.05 ml of 0.2 *N* methanolic potassium hydroxide. After 20 minutes at 37° a drop of water was added followed by equivalent Dowex-50·H^+ for neutralization. The resin was centrifuged. Chromatograms of the solution revealed one product. Its R_f was 0.1 in both PW and BPAW and it readily eluted with water. Paper electrophoresis demonstrated its strong anionic nature Acid hydrolysis produced the $R_f = 0.05$ compound. A sample of the sulfolipid-C^{14} was deacylated similarly and gave 14,200 cpm fatty acid and 5,850 cpm of the $R_f = 0.1$ product, a ratio of 21.7 to 9.

Presence of free hydroxyl groups: Pyridine solutions of the S^{35}-labeled hydrolysis products were treated with acetic anhydride and chromatographed. The presence of free hydroxyl groups was revealed by the high R_f values of the products. Free hydroxyl groups in the lipid-S^{35} were established by benzoylation with benzoyl chloride in dry pyridine during two hours at room temperature. Chromatography of the solution revealed formation of a new compound with $R_f = 0.9$ in both PW and BPAW. This compound was deacylated by treatment with 0.1 *N* methanolic potassium hydroxide at 37° for 45 minutes. The single product had R_f values of 0.6 in PW and 0.5 in BPAW. Treatment with 2 *N* methanolic potassium hydroxide at 75° for two hours gave a product with $R_f = 0.1$ in both solvent systems. Its properties were identical with that directly derived by deacylation of the lipid-S^{35}.

Acid hydrolysis products of sulfolipid-C^{14}: The C^{14}-labeled sulfolipid was hydrolyzed in 2 *N* hydrochloric acid for 30 minutes at 100°. The hydrolysate was chromatographed to provide the radiogram shown in Figure 2. The products were 2,100 cpm glycerol, 13,600 cpm fatty acid, and 4,370 cpm of the $R_f = 0.05$ compound. This is a ratio of 3:18.7:6.2.

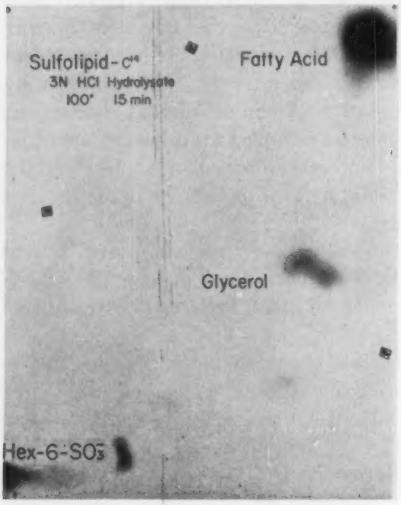

FIG. 2.—Radiogram of acid hydrolysis products of sulfolipid-C^{14}.

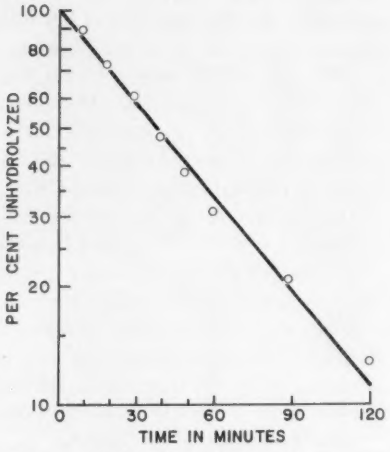

FIG. 3.—Acid hydrolysis rate of 1′-glyceryl glycoside-6-sulfonate in *N* hydrochloric acid at 100°.

The glycerol-C^{14} produced was identified by R_f value and by periodate oxidation which yielded formic acid and formaldehyde. These were isolated as barium formate and formaldimedone in a C^{14} activity ratio of 0.95 to 2.0.

The $R_f = 0.05$ product was then cochromatographed with synthetic galactose-6-sulfate prepared according to the method of Percival and Soutar.[6,7] It cochromatographed closely with the unknown in PW, BPAW, and butanol-pyridine-water solvents.

Identification of aldohexose‡ structure: With the presence of six carbon atoms and hydroxyl groups indicated, the $R_f = 0.05$ compound was treated with 2,4-dinitro-

phenylhydrazine to produce two products with increased R_f values corresponding to a hydrazone and an osazone.

Treatment of the sugar sulfonate with 3 per cent concentrated hydrochloric acid in ethanol at 100° for 15 hours produced an ethyl glycoside sulfonate with R_f = 0.35 in PW. The glycoside was completely hydrolyzed in normal hydrochloric acid at 100° in thirty minutes. After ten hours' heating without apparent decomposition the sugar sulfonate could be reconverted to the R_f = 0.35 ethyl glycoside sulfonate. Tritylation of the R_f = 0.05 compound or its derivatives was unsuccessful.

Properties of glyceryl glycoside-6-sulfonate: The deacylated lipid was resistant to bromine oxidation during six hours at room temperature in the presence of barium carbonate. Under these conditions, the sugar-sulfonate-S^{35} was oxidized to form a new compound with R_f = 0.1 in PW. Further, the sulfolipid-C^{14} was deacylated to give fatty acid and water-soluble product in a ratio of 18 to 8. The same water-soluble product hydrolyzed in acid to give 70 cpm of glycerol and 147 cpm of sugar-6-sulfonate, a ratio of 2.8 to 6.0.

Acid hydrolysis rate of glyceryl glycoside-6-sulfonate: The acid hydrolysis rate of the sulfur-labeled compound was determined by chromatography of aliquot portions as a function of time in mineral acid. Its rate of acid hydrolysis ($T_{1/2}$ = 30 minutes in *N* hydrochloric acid at 100°) is shown in Figure 3.

Nature of the glycosidic linkage: The glycoside-C^{14} was stable to the α-galactosidase of bottom yeast invertase,[8] an enzyme capable of hydrolyzing the isomeric 2-glyceryl α-D-galactoside (floridoside) which occurs in red algae.[9] Solution of glyceryl glycoside-6-sulfonate-S^{35} and 2 mg. of β-galactosidase-containing emulsin in 0.1 ml of water at pH 5 with a drop of toluene produced a 70 per cent yield of sugar-6-sulfonate-S^{35} after five days at room temperature.

Periodate oxidation of glyceryl glycoside-6-sulfonate: The position of the glycosidic linkage on a primary hydroxyl of glycerol was established by periodate oxidation of the glyceryl glycoside-6-sulfonate-C^{14} to yield one mole of formaldehyde-C^{14}. To 12,500 cpm of carbon-labeled glyceryl glycoside-6-sulfonate in 0.10 ml of water were added 3.0 mg. of glycerol in 1.5 ml of water and 0.25 ml of 0.5 *N* periodic acid. The solution was allowed to stand five hours at room temperature and was then treated by the procedure used in the glycerol degradation to produce formaldimedone. After correction for the yield from 3.0 mg. glycerol, the C^{14} activity of the formaldimedone was 1,550 cpm. The theoretical yield of formaldehyde from 12,500 cpm of a 1′-glyceryl glycoside-6-sulfonate is 1,400 cpm. The compound, therefore, differs from the 2-glyceryl galactoside of red algae and is related to those of the galactolipids, recently isolated by Carter, McCluer, and Slifer,[10] which are 1-glyceryl β-D-galactoside esters.

Glyceryl glycoside-6-sulfonate-C^{14} (90,400 cpm) and 31.4 mg glycerol in 2.4 ml water were oxidized with 1.0 mmol. sodium periodate at pH 6 for two hours.[11] The volatile products were distilled *in vacuo*. The distillate was neutralized with barium hydroxide to give barium formate with a specific activity of 180 cpm/mg after recrystallization. This indicated that 0.9 mole of formic acid was produced from one mole of the glycoside sulfonate. This result suggests an aldopyranoside-6-sulfonate structure.

Fatty acid of the Chlorella sulfolipid: It appeared from the results of hydrolyses of the sulfolipid-C^{14} that there is only one fatty acid present. The fatty acid-C^{14}

has been shown[12] to be largely oleic acid by paper cochromatography with oleic acid and hydrogenation to stearic acid-C^{14}. The location of the oleoyl ester on the 3-position of glycerol was established in the following manner. A sample of the sulfolipid-C^{14} was benzoylated in pyridine and the product isolated by chromatography in PW and BPAW. It was deacylated by treatment with 0.1 *N* methanolic potassium hydroxide at 37° for 30 minutes. Under these conditions benzoyl groups[13] are not removed. The hexose-6-sulfonate was removed by treatment with *N* hydrochloric acid at 100° for 90 minutes. Benzoyl glycerol was isolated by paper chromatography of the hydrolysate and subjected to periodate oxidation in the presence of glycerol carrier. No C^{14} activity was present in the formaldimedone produced.

Discussion.—The experimental results strongly suggest the structure shown in Figure 4 for the plant sulfolipid. The fact that it is a glycosyl monoglyceride establishes its relationship to the galactolipids recently identified among wheat flour lipids by Carter, McCluer, and Slifer.[10] It is clear from the present work that there is indeed only one fatty acid in the molecule and that the galactolipids are monoglyceride derivatives.

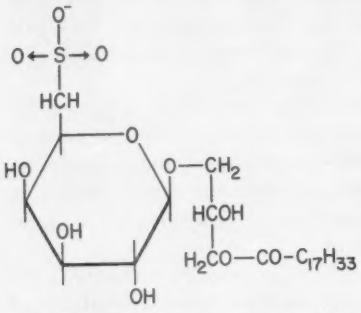

FIG. 4.—Proposed structure for the plant sulfolipid.

The resistance of the anionic S^{35}-labeled hexose derivative to acid hydrolysis suggests the presence of a sulfonic acid group. Production of formic acid by periodate oxidation of its glycoside implies that it may be a 6-sulfonic acid derivative of a 6-deoxyhexose. While R_f values are nearly identical, galactose 6-sulfate was readily hydrolyzed by acid whereas the unknown sugar sulfonate was not. Since no synthetic α-hydroxy sulfonic acids appear to have been reported, complete structural assignment must await isolation and synthesis.

A sulfonic acid group in the sulfolipid would be unique in carbohydrate chemistry. The biochemical implications of such a compound are intriguing. The most reasonable mode of biosynthesis would be via peroxide oxidation of an -S-S- linked thioglycolipid. Of all metabolic structures the photosynthetic apparatus is the most likely to provide such an oxidative environment.

The apparent ubiquity of the sulfolipid within green plant tissues, its high concentration, and its rapid metabolism[14] imply that it performs physiological and chemical functions at least as important as those of the phosphatides. The strong anionic properties of the sulfonate ion could provide oriented polar interfaces potentially useful in the photosynthesis process. Its counterpart in nervous tissues, cerebroside sulfate,[15] has similar solubility properties and perhaps similar physico-chemical functions.

Summary.—A sulfolipid occurs in high concentrations in photosynthetic tissues. It is readily labeled with S^{35} or C^{14} during photosynthesis by *Chlorella*. Radiochromatographic examination of the sulfolipid molecule revealed an anionic aldohexopyranosyl monoglyceride structure. Acid hydrolysis gave an acid-stable anionic hexose containing the sulfur. Periodate oxidation of the ethyl glycoside-

sulfonate-C^{14} produced one mole of formic acid. The structure of the sulfolipid consistent with the observed properties is 1-O-(β-6′-deoxy-aldohexopyranosyl 6′-sulfonic acid)-3-O-oleoyl glycerol. The sulfonic acid structure suggests its origin by peroxidation of an -S-S- linked glycolipid in the photosynthetic apparatus.

The authors appreciate the helpful suggestions of Professors J. K. N. Jones of Queens University, B. Maruo of Tokyo University, and M. L. Wolfrom of Ohio State University.

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† Part of the material in this paper is included in the thesis submitted by R. Wiser to the Graduate School of The Pennsylvania State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy, August, 1958.

‡Available structural evidence is also consistent with an isomeric 1-deoxyketopyranoside 1-sulfonic acid structure.

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*AN EFFECT OF AUXINS ON THE HEAT COAGULABILITY OF THE PROTEINS OF GROWING PLANT CELLS**

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Communicated by J. S. Fruton, September 22, 1959

Most current theories of auxin action are based on the discovery by Heyn[1] that the application of auxin to growing plant cells results in an increased plastic extensibility of the cell wall. While such a mechanism suffices to explain elongation induced by auxin, it is obviously inadequate to account for the known effects of

auxin on the cytoplasm[2-8] and the nucleus,[9] or to explain the mitogenic or morphogenetic aspects of auxin action.

We have recently conducted experiments with C^{14}-carboxyl-labeled 2,4-dichlorophenoxyacetic acid (2,4-D) fed to growing pea stem sections in an attempt to localize the region of the cell in which auxin acts. A centrifugal fractionation of the cell contents after several hours of auxin-induced growth revealed the label not to be attached to any cell organelle, but rather to be localized in the final centrifugal supernatant fraction devoid of all particulates. When we attempted to precipitate the proteins in this supernatant fraction to determine whether any of the labeled auxin was protein bound, we discovered fortuitously that the proteins derived from cells treated with the auxin failed almost completely to coagulate after 10 minutes of boiling, while proteins of the control cells produced a copious white precipitate under the same conditions. Repetition of this experiment with various auxins and analogs, and with quantitative measurement of the precipitate, revealed a close correlation to exist between the effect produced on the protein and the effect produced on growth. We thus consider that this phenomenon, which we interpret as an auxin-induced alteration of the physical state of the cellular proteins, may be important in explaining auxin action.

Materials and Methods.—Alaska peas, obtained from Associated Seed Growers, New Haven, Connecticut, were soaked in tap water for three hours and sown in water-saturated vermiculite in polyethylene containers. They were then grown in darkness for 7 days at a temperature of 27° ± 1°C. At the end of this time, 10 mm-long subapical stem sections of the third internode were excised with a double-bladed cutting tool and used for the overnight incubations. Approximately 80 such sections (the number varied slightly between experiments) were placed in 10 ml of growth medium in a 9-cm petri dish and incubated overnight (*ca.* 18 hr) in the dark room. The incubation medium contained 2 per cent sucrose, 0.02 *M* pH 6.1 phosphate buffer and the auxin or auxin analog, where added. The growth of the sections was estimated by length and fresh weight measurements.[10] After harvest, the sections were rinsed, homogenized in a pre-chilled mortar and pestle with 14 ml of cold 0.5 *M* sucrose + 0.001 *M* ethylenediaminetetraacetic acid (EDTA), strained through four layers of washed cheesecloth, and centrifuged at 24,000 × *g* for one hour in a water-cooled Sorvall centrifuge in the cold room. The final volumes of the decanted supernatant liquids were then equalized with fresh sucrose-EDTA (the total volume was usually 19 ml) and appropriate aliquots then removed for heat coagulation, for precipitation with an equal volume of 1 *M* trichloroacetic acid (TCA), and for other purposes. The heat coagulation was achieved by placing 5-ml aliquots in test tubes immersed in a boiling water bath, usually for 10 minutes. At the end of this time, differences in turbidity were clearly apparent between control and auxin-treated proteins, the former being much more turbid. Where no obvious precipitates formed, the turbidities were measured in a Klett colorimeter equipped with a #42 blue filter. Where precipitates formed, they were centrifuged down, usually washed twice with 5-ml aliquots of distilled water, then pipetted onto tared weighing dishes, dried overnight in an oven and weighed.

In certain experiments, 14–16-day-old light grown peas (16-hr photoperiod, 1500 ft. candles intensity of mixed fluorescent and incandescent light, temperature

23°C) were used as a source of tissue. In this instance, about 160 five-mm stem sections (or half the number of 10-mm sections) were used per dish and the sections were incubated overnight in the light.[11]

Results.—Table 1 indicates typical results obtained with etiolated pea tissue material. The total protein, as obtained by TCA precipitation, is not altered by auxin treatment, but the fraction precipitable by boiling for 10 min is sharply reduced by the 2,4-D.

Table 2 shows a comparison of the activity of 2,4-D and of several other auxins and auxin analogs. Clearly, the most active auxins (IAA and 2,4-D) produce

TABLE 1

THE EFFECT OF 2,4-D ON THE PRECIPITABILITY OF ETIOLATED PEA STEM PROTEINS

2,4-D Molarity	Dry wt(mg) of Twice-Washed Precipitate	
	TCA Method	Heat Method
0	5.9	4.6
10^{-5}	5.8	0.4

TABLE 2

EFFECT OF VARIOUS AUXINS AND AUXIN ANALOGS ON THE GROWTH AND HEAT COAGULABILITY OF PROTEINS OF ETIOLATED PEA EPICOTYL SECTIONS. ALL SUBSTANCES TESTED AT 10^{-5}M

Substance	Per cent Increase in		Dry wt. (mg) of Twice Washed Heat Coagulated Protein
	Length	Fresh wt.	
Control (no addendum)	50	58	10.1
2,3,5-triiodobenzoic acid (TIBA)	40	54	6.3
2,4-D	82	106	1.2
p-Cl phenoxyisobutyric acid (PCIB)	42	45	9.9
Phenylacetic acid (PAA)	62	51	6.4
Indoleacetic acid (IAA)*	85	108	1.8

*Separate experiment.

TABLE 3

THE EFFECT OF VARIOUS CONCENTRATIONS OF 2,4-D ON THE GROWTH AND PROTEIN COAGULABILITY OF GREEN AND ETIOLATED PEA STEM SECTIONS

2,4-D Molarity	Green Sections			Etiolated sections	
	% Increase in Fresh wt.	Turbidity Reading	Mg Dry wt of Unwashed Heat-Coagulated Protein	% Increase in Fresh wt.	Mg Dry wt of Unwashed Heat Coagulated Protein
0 (initial control)	..	113	37.7	..	65.3
0 (final control)	33	136	27.5	55	53.6
10^{-6}	67	122	28.7	114	40.3
10^{-5}	93	87	20.1	130	21.9
10^{-4}	86	56	17.6	124	18.4

the greatest reduction in the weight of the protein coagulum; PCIB, which is an antiauxin analog of 2,4-D and slightly inhibitory to growth, produces no change in the protein precipitated; TIBA and PAA, both known to be slightly active as auxins,[12] produce smaller effects on the proteins.

Table 3 shows the effect of various concentrations of 2,4-D on the turbidity and heat coagulability of the proteins of green and etiolated pea stem sections. Clearly, there is an increasing effect on the proteins with increasing concentration of 2,4-D, even where the growth optimum may have been surpassed.

Subsequent experiments have revealed that: (1) 2,4-D is practically inactive when applied *in vitro* to control proteins, only slight effects being produced by the highest concentration (10^{-4} *M*) applied, (2) the difference between control and treated tissues resides mainly in the proteins of the non-particulate fraction, since boiling of the uncentrifuged homogenate yields differences only slightly greater than with all the particles removed, (3) the effect of 2,4-D on the proteins of etiolated pea stems can be detected in as little as four hours, and (4) in pea roots, where auxins induce marked growth inhibitions, effects similar to those described above are produced by overnight incubation with as little as 10^{-8}*M* 2,4-D.

Discussion.—It is at present impossible to estimate the physiological importance of the phenomenon described. A decision on this question will have to be deferred until the completion of extensive and lengthy tests on the relation of structure of auxin-type molecules to activity, on the relation of concentration of active molecules to activity, and on further kinetic experiments.

Whether or not the phenomenon is physiologically meaningful, it would appear to have considerable interest for the protein chemist. For example, it would be interesting to know whether the auxins produce this effect by attachment to the proteins, as previously indicated,[13] or by some indirect mechanism.

This phenomenon could explain the auxin-induced decreased viscosity of the cytoplasm described by Northen[2, 3] and the effects on cytoplasmic streaming observed by Sweeney and Thimann.[4–8] It might also be part of a general phenomenon which would explain the effects of auxin on wall[1] and nucleus,[9] as well as on the cytoplasm.

Summary.—Auxins induce a decrease in the heat coagulability of the proteins of growing pea stem cells to which they have been applied. The total protein content is not altered. The effect is produced mainly in the non-particulate phase of the cytoplasm. Auxin analogs which do not promote growth are less effective or completely ineffective. The effect is greatly reduced, or not produced at all when the active molecules are added *in vitro*.

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ANOMALOUS OPTICAL ROTATORY DISPERSION OF DYE: POLYPEPTIDE COMPLEXES[*,†]

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The optical rotatory dispersions of various anionic azo dye:serum albumin complexes outside the region of the dye absorption maxima have been reported to be significantly different from those of serum albumin solutions without dye. Since the dyes alone possessed almost no optical rotatory activity, the observed differences were thought to be the result of structural changes occurring in the serum albumin molecule upon the binding of dye.[1]

To investigate the various factors influencing the optical rotation of dye:protein complexes, we have initiated a study of the optical rotatory dispersion of complexes of dyes and high molecular weight water-soluble polypeptides of known structure and conformation. In this communication we describe the results with two cationic dyes and poly-α,L-glutamic acid (L-PGA).

Experimental.—Commercial preparations of dyes were used: Acriflavine Neutral (National Aniline) and Rhodamine 6G (E. I. du Pont). The sodium salt of poly-α,L-glutamic acid, sample R4273–112, prepared in the manner previously described[2] was used to prepare the free acid (L-PGA). This sample has an intrinsic viscosity of 1.12 at pH 7.3 in 0.2 *M* NaCl from which a weight average molecular weight of 51,000 is estimated. A Rudolph spectropolarimeter with a mercury arc was used, and readings were taken at 23°C (±2°). 1-mm, 1-cm, and 10-cm cells were used. The dye:PGA solutions were prepared and adjusted to pHs between 4.5 and 4.9 by slow addition of 0.1 *N* and 1 *N* HCl with constant stirring. The clear solutions were immediately used and were protected from light before measurements were made.

Results.—The dyes alone showed no optical rotation. The optical rotatory dispersion of L-PGA alone in its helical conformation at pH 4.8 is shown in Figure 1 alone with that of the Acriflavine:L-PGA complex. This complex and the other (Table 1) show markedly anomalous optical rotatory dispersions in the wavelength regions corresponding to the dye absorption bands.

TABLE 1

Anomalous Optical Rotation in the Absorption Band of Cationic Dyes bound to Polypeptide

Dye:L-PGA Complex and pH	Dye Concentration	PGA Carboxyl Concentration	Carboxyl/Dye Ratio	Inflection Point of Anomalous Rotatory Dispersion	Absorption Maximum of Bound Dye
Acriflavine pH 4.9	3.3×10^{-4} *M*	6.1×10^{-2} *M*	185	458 mμ	457 mμ
Rhodamine 6G pH 4.6	2.5×10^{-5} *M*	6.6×10^{-2} *M*	2640	491, 502, 533 mμ	496 and 529 mμ
	5.0×10^{-5} *M*	6.6×10^{-2} *M*	1320	492, 506, 537 mμ	498 and 530 mμ

The observed anomalous rotatory dispersion of the dye:PGA complexes is the Cotton effect[3] (circular dichroism). The Cotton effect observed in these complexes indicates that the chromophoric group of the dye has acquired asymmetry, since a

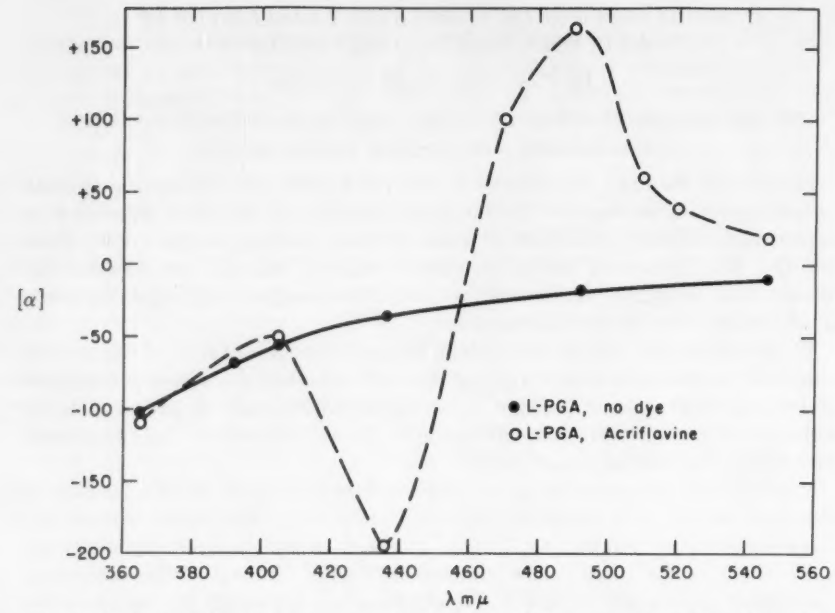

FIG. 1.—Optical rotatory dispersion of an Acriflavine:L-PGA complex at pH 4.9. Concentrations are Acriflavine, 3.3×10^{-4} *M*; PGA carboxyl, 6.1×10^{-2} *M*. [α] for both figures has been calculated an the basis of PGA concentrations.

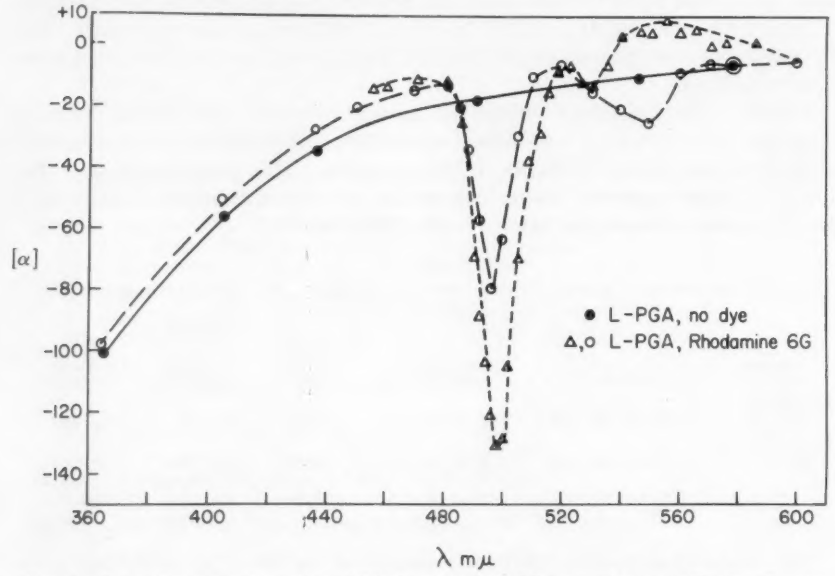

FIG. 2.—Optical rotatory dispersion of Rhodamine 6G:L-PGA complexes at pH 4.6. Concentrations are Rhodamine 6G, –○–○–, 2.5×10^{-5} *M* and –Δ–Δ–, 5×10^{-5} *M*; PGA carboxyl, 6.6×10^{-2} *M*.

symmetrical dye molecule does not show this effect.[4] This finding is of considerable interest; we believe that this is the first report that the chromophoric group of a symmetrical molecule may show this effect upon binding to an asymmetric helical macromolecule.

The inflection points in the optical rotatory curves indicate the absorbing moiety responsible for the anomaly inasmuch as these inflections show good agreement with the absorption maxima of the dyes (Table 1). Rhodamine 6G:L-PGA solutions exhibit several inflection points in its dispersion curves (Fig. 2). Two of these are near absorption maxima observed at 496 and 529 mμ.

In contrast to results obtained when the L-PGA is in the helical conformation at low pH, no anomalous dispersion is exhibited by the dye:PGA complexes at pHs above 6, where the PGA is in a random conformation.[2, 5] L-PGA in the random conformation strongly binds the dyes, as shown by equilibrium dialysis and spectral shift studies, and thus the difference appears to be related to the helix content. The magnitude of the Cotton effect of Acriflavine:L-PGA as a function of pH shows good agreement with the helix content as determined from $[\alpha]_{546}$.

It should be noted that a large Cotton effect is observed with low dye concentrations. $[\alpha]$ for all figures has been calculated on the basis of PGA concentration. However, on the basis of dye concentrations, the observed anomalous rotations would be of the order of 20,000. The general nature of this effect has been confirmed and extended by experiments with several other cationic dyes.[6]

In summary, cationic dyes and helical PGA interact and apparently create an asymmetry in the chromophoric groups of the formerly symmetrical dye as evidenced by the observed Cotton effect. The dependence of the observed circular dichroism on the helix content of the polypeptide may make this a useful method in estimating the helix content of other polypeptides and proteins. In order to further study this effect experiments are underway with polypeptides of the opposite sense of twist of the helix as well as investigations of dye interactions with other types of helical macromolecules. The results reported here thus demonstrate that interpretations of optical rotatory data must take into account the possibility that an optically inactive substance may contribute to the optical rotation upon binding to an asymmetrical substance, and indicate that this phenomenon may be utilized as a tool in the study of interactions between small molecules and macromolecules.

We wish to thank Mr. Kenneth Norland for numerous stimulating discussions concerning this work.

* This is *Polypeptides XXVI*. For the preceding paper in this series see Bird, G. R., and E. R. Blout, *J. Am. Chem. Soc.*, **81**, 2499 (1959). (Alternate address of E. R. Blout, Chemical Research Laboratory, Polaroid Corporation, Cambridge 39, Massachusetts.)

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SIGNIFICANT STRUCTURES IN LIQUIDS. II.

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In the first paper of this series,[1] a procedure was described for arriving at a model of the liquid state. The procedure identifies three significant structures in the liquid state. First, solid-like degrees of freedom result from binding of a molecule to an equilibrium position by its neighbors. The second significant structure arises from possible position degeneracy in the solid-like structure. If additional vacant sites are available to a molecule in addition to its equilibrium position, some strain energy is stored in the lattice as the molecule shifts position. Finally, gaslike degrees of freedom occur as a molecule escapes in at least one direction from the solid lattice.

Bonding energy is attributed to solid-like degrees of freedom. The concentrations of the significant structures are estimated in the following way. The bonding energy is taken as $E_s(V_s/V)$, where E_s is the energy of sublimation of the solid at the melting point, V_s is the molar volume of the solid at the melting point, and V is the liquid volume. Then V_s/V is assumed to be the fraction of solid-like degrees of freedom and $(V-V_s)/V$ is the gaslike fraction.

The partition function, f, of a normal liquid such as argon is then

$$f = \left[\frac{e^{\frac{E_s}{RT}}}{\left(1-e^{-\frac{\theta}{T}}\right)^3} \left(1 + n_h\, e^{-\frac{a}{n_h RT}}\right) \right]^{N\frac{V_s}{V}} \left[\frac{\left(2\pi\, mkT\right)^{\frac{3}{2}}}{h^3} \frac{eV}{N} \right]^{N\frac{(V-V_s)}{V}} \tag{1}$$

In the above, n_h is the number of equilibrium sites accessible to a molecule in addition to the single most stable position. a/n_h is the strain energy stored in the system as the molecule shifts to one of the n_h sites. θ is the Einstein characteristic temperature. The two new parameters characteristic of the liquid state are the extra sites per molecule, n_h, added in the liquid and the strain energy, a/n_h, required to occupy these sites.

For the energy of distortion of argon:

$$a/n_h = 0.00534\, E_s\, V_s/(V - V_s) \tag{2}$$

The linear dependence of strain energy on the inverse of the concentration of holes is a reasonable relationship.

The partition function (1) permits the calculation of all thermodynamic properties for normal liquids.

The first square brackets of Equation (1) enclose that part of the partition function to be attributed to solid-like degrees of freedom. Gaslike degrees of freedom are represented by the expression inside the second set of square brackets. The gaslike part of the partition function in the general case contains f_{vib} and f_{rot} for any internal vibration or rotational degrees of freedom which the molecule may possess. If the motion is present in the solid state also, the appropriate terms appear as a factor in the over-all expression.

The parameters n_h and a have been studied to see how they vary from liquid to liquid when the best fit between calculated and experimentally observed properties

is obtained. n_h is calculated by $n\ (V - V_s)/V_s$. The pressure effect on viscosity would predict a value for n of about 7. It is probable that a temperature variation of θ would allow $n = 7$, but this study takes θ as constant since such a temperature variation is not theoretically calculable at present. The quantity, a, is related to E_s by the relation

$$\frac{a}{n_h} = \frac{aE_sV_s}{n\,(V - V_s)} = \frac{a'\,E_sV_s}{V - V_s}.$$

In Tables 1 and 2, the values of n and a' and the solid parametric values, respectively, are listed which were used in this paper.

TABLE 1

VARIATION OF PARAMETERS

	n	a'
Neon	11	0.00802
Argon	10.8	0.00534
Krypton	11.7	0.00590
Xenon	12.27	0.00563

TABLE 2

SOLID PARAMETERS USED

	Neon	Argon	Krypton	Xenon
Melting point, °K	24.55	83.85	116.0	161.3
E_s, cal.	447.4	1,888.6	2,710.4	3,897.7
V_s, ml	13.98	24.98	28.4	36.5
θ, °K	44.7	60.0	45.0	39.2

The calculation of the best fitting values of n and of a' produced calculated properties of the rare gases differing slightly from those reported earlier.[1,2] The results obtained are compared with experiment in Table 3.

TABLE 3

CALCULATED AND OBSERVED PROPERTIES

	Neon			Argon			Krypton			Xenon		
	Calc.	Obs.	Δ, %	Calc.	Obs.	Δ, %	Calc.	Obs.	Δ, %	Calc.	Obs.	Δ, %
V_m	16.43	16.15	+1.71	28.90	28.03	+3.11	33.11	34.13	−2.98	42.30	42.68	−0.88
ΔS_m	3.244	3.259	−0.44	3.263	3.35	−2.61	3.456	3.35	+3.16	3.415	3.40	+0.44
P_m	0.437	0.417	+4.81	0.732	0.674	+8.55	0.756	0.722	+4.68	0.399	0.804	−50.4
V_b	17.17	16.80	+2.19	29.33	28.69	+2.22	...	...	...	...	...	...
ΔS_b	15.68	15.81	−0.83	19.04	17.85	+6.68	19.27	17.99	+7.13	19.43	18.29	+6.24
T_b	27.13	27.26	−0.48	87.29	87.29	0	119.28	119.93	−0.54	167.5	165.1	+1.45

Here, V_m, ΔS_m, and P_m are the liquid volume, the entropy change, and the vapor pressure at the melting point, respectively. V_b, ΔS_b, T_b are the analogous properties at the normal boiling point.

Application of the partition function to more complicated molecules was made. Nitrogen, methane, and benzene were studied with the results shown in Tables 4, 5, and 6. Here, the parametric values, which were used in the calculations, are given in the beginning of each table.

In the benzene calculation, two values of the Einstein θ were required; one for the solid and another, θ', for the solid-like part of the liquid partition function. This result, and the benzene calculation in general, is in good agreement with that reported earlier by Walter and Eyring.[3]

TABLE 4

METHANE

Melting point, °K	90.65	n	13.3
E_s, cal	2200	a'	0.00296
V_s, ml	30.94	θ	75.33
ΔS, fusion, e.u.	2.48		

	V			Vapor Pressure		
T°K	Calc.	Obs.	Δ, %	Calc.	Obs.	Δ, %
90.65(T_m)	34.111	33.63	+1.43	0.1172	0.115	+1.95
99.67	35.024	...	...	0.3265	0.3282	−0.51
111.67(T_b)	36.354	37.816	−3.86	0.9736	1.0000	−2.64
112	36.447	...	...	1.0054	...	...

ΔS_{vap}, e.u.			T_{vap} at 1 atm. °K		
Calc.	Obs.	Δ, %	Calc.	Obs.	Δ, %
17.399	17.51	−0.63	112.0	111.67	+0.29

Critical Properties	Calc.	Obs.	Δ, %
P_c, atm	63.305	45.8	+38.2
V_c, ml	103.90	99.02	+4.30
T_c, °K	214	191.04	+12.0

TABLE 5

NITROGEN

Melting point, °K	63.14	n	12.9
E_s, cal	1529.9	a'	0.00343
V_s, ml	29.31	θ	55.94
ΔS, fusion, e.u.	2.73		

	V, ml			Vapor Pressure, atm		
T°K	Calc.	Obs.	Δ, %	Calc.	Obs.	Δ, %
63.14(T_m)	32.622	31.95	+2.10	0.1244	0.1237	+0.55
68.41	33.560	33.09	+1.42	0.3014	0.3005	+0.29
77.34(T_b)	35.055	34.70	+1.02	0.9945	1.0000	−0.55
99.52	41.034	40.51	+1.29	7.17	7.37	−2.7

ΔS_{vap} = 17.433 e.u. calc., 17.24 e.u. obs., +1.12%

Critical Properties	Caled.	Obs.	Δ, %
P_c, atm	40.471	33.49	+20.85
V_c, ml	98.482	90.1	+9.30
T_c, °K	135.3	126.0	+7.38

TABLE 6

BENZENE

Melting point, °K	278.6	n	20
E_s, cal	10,383.8	a'	0.006
V_s, ml	77.00	θ	80.24
ΔS, fusion, e.u.	8.46	θ'	55.2

	V, ml			Vapor Pressure, atm		
T°K	Calc.	Obs.	Δ, %	Calc.	Obs.	Δ, %
278.6(T_m)	87.01	87.28	−0.31	(0.0474)	0.0474	...
298.1	88.94	89.36	−0.47	0.1261	0.124	+1.50
328.1	92.01	92.80	−0.85	0.4337	0.4293	+1.03
353.3(T_b)	94.94	95.92	−1.06	1.007	1.0000	+0.74
373.1	97.25	98.46	−1.23	1.724	1.757	−1.91
423.1	104.72	106.8	−1.95	5.738	5.704	+0.60

ΔS_{vap} = 21.311 e.u. calc., 20.848 e.u. obs., +2.22%

Critical Properties	Calc.	Obs.	Δ, %
P_c, atm	74.39	47.9	+55.3
V_c, ml	268.7	256	+4.97
T_c, °K	644	562	+14.6

A method of procedure for more complicated molecules such as ethane is to use the following expression as the solid part of the liquid partition function:

$$\left[\frac{e^{\frac{E_s}{RT}}}{\left(1-e^{-\frac{\theta_1}{T}}\right)^3}\left\{\frac{1}{1-e^{-\frac{\theta_2}{T}}}\left(1-e^{-\frac{\delta E_s}{(V-V_s)RT}}\right)+f_{1,\,\mathrm{rot}}\,e^{-\frac{\delta E_s}{(N-V_s)RT}}\right\}\times\left(1+n_h e^{-\frac{a}{n_h RT}}\right)\right]^{\frac{NV_s}{V}} \quad (3)$$

The curled brackets enclose the partition function for an oscillator which gradually turns to a free rotator at higher temperatures. This function was not given extensive testing since the necessary data for solid ethane were not available. Here, δE_s is an activation energy for free rotation, δ being a fractional number.

At the critical point, the liquid partition function (1) is seen to predict values which differ substantially from those observed (Table 7).

TABLE 7

CRITICAL DATA FOR ARGON

	Calc.	Obs.	Δ, %
T_c, °K	149.7	150.66	−0.637
V_c, ml	83.68	75.26	+11.2
P_c, atm	52.93	48.00	+10.3

It was felt that the pressure would be lowered if the partition function reflected the clustering of molecules in the gas phase at the critical point. Accordingly, the partition function becomes

$$f=\left\{\frac{e^{-\frac{E_s}{RT}}}{\left(1-e^{-\frac{\theta}{T}}\right)^3}\left[1+n_h e^{-\frac{a}{n_h RT}}\right]\right\}^{\frac{NV_s}{V}}\left\{\left[\frac{(2\pi mkT)^{\frac{2}{3}}}{h^3}\frac{eV}{N}\right]^2+\frac{(2\pi(2m)kT)^{\frac{3}{2}}}{h^3}\frac{eV}{\frac{N}{2}}\frac{\left(e^{\frac{D}{RT}}-1\right)}{1-e^{-\frac{\theta}{T}}}\frac{8\pi^2IkT}{2h^2}\right\}^{\frac{V-V_s}{V}\frac{N}{2}} \quad (4)$$

for a liquid such as argon with no ordinary internal degrees of freedom. The contribution made by diatomic gas molecules is seen to depend upon the size of the coefficient $(e^{\frac{D}{RT}}-1)$. This factor corrects for the energy difference between the monatomic and diatomic gas molecules. I is the moment of inertia of the diatomic molecule. If the vibration frequency is taken equal to the lattice vibration frequency and the two atoms in the molecule are assumed to be separated by a distance of two van der Waals radii, then the dissociation energy of the diatomic molecule, D, is the only arbitrary number. Various values of D were chosen and the differences, Δ, between calculated and observed values of the critical point data were obtained. It was found that the pressure may indeed be lowered in this way but that the value of Δ for the critical volume now is too large.

The results we have presented here show that the method of significant structures may be applied to more complicated molecules than those treated in an earlier paper.[1]

APPENDIX

(1) *Partition Function for the Solid-like Part of Liquids.*—Experimental evidence, e.g., the curve of specific heat versus temperature, and simple theoretical

calculations of volume indicate that methane and nitrogen molecules rotate in the solid state. Thus in the calculations of Table 4 and 5, the following formulas, respectively, were used for the solid part of the liquid partition function

$$(1 - e^{-\theta/T})^{-3}\, e^{\frac{E_s}{RT}}\, \frac{\pi^{1/2}}{12} \left(\frac{8\pi^2 IkT}{h^2} \right)^{3/2} \prod_{i=1}^{9} \frac{1}{1 - e^{-h\nu_i/kT}} \tag{5}$$

and

$$(1 - e^{-\theta/T})^{-3}\, e^{\frac{E_s}{RT}}\, \frac{8\pi^2 IkT}{2h^2}\, \frac{1}{1 - e^{-h\nu/RT}} \tag{6}$$

Here, I is the moment of inertia and ν is the internal frequency of the molecule. These molecular parameters are considered to be equal to those for gas molecules, and the data found in the literature[4] were used in the calculations.

For the solid part of the liquid partition function of benzene, we used the following expression:

$$(1 - e^{-\theta/T})^{-6}\, e^{\frac{E_s}{RT}} \prod_{i=1}^{30} \frac{1}{1 - e^{-h\nu_i/kT}} \tag{6}$$

Here, it is assumed that a three-dimensional torsional vibrator oscillates with the frequency which is equal to that for the lattice vibrator.

(*2*) *Determination of E_s and θ.*—The θ values in the solid-like part of the liquid partition function of complex molecules are in general the Einstein characteristic temperatures fitted to the solid at the melting point. These values do not necessarily agree with the literature values which are usually the values obtained at temperatures considerably below the melting point. The procedures, by which the values θ and E_s were obtained, are given below.

For methane, the solid vapor pressure data[5] with the Clapeyron-Clausius equation gives the value of E_s used. Using the equilibrium condition between gas and solid at the melting point, i.e.,

$$F_{\text{solid}} = F_{\text{gas}}$$

We obtain

$$A_{\text{solid}} \simeq A_{\text{gas}} + RT_m \tag{7}$$

Here, it has been assumed that the PV product for the solid is negligibly small and that the gas is ideal (which is reasonable for the low vapor pressure observed at the melting point). Now A_{gas} is readily calculated from the partition function. Then

$$A_{\text{solid}} = -RT_m \ln f_{\text{solid}} \simeq A_{\text{gas}} + RT_m \tag{8}$$

where f_{solid} is given by expression (5). It is seen that θ is now the only unknown quantity in (8) and can be readily calculated.

An alternate method is illustrated by the determination of E_s and θ for the nitrogen calculation. Use was made of the solid entropy, calculated by graphical integration of the (C_p/T) vs. T curve. This entropy, if equated to that calculated from the partition function by $S = -(\partial A/\partial T)_v$, allows an approximation of the value of θ, since E_2 does not appear in the entropy expression.

Admittedly, the two results differ by

$$\int_0^{T_m} \frac{C_p - C_v}{T}\, dT$$

but the correction will be small. Once θ is estimated in this way, equations (7) and (8) give the value of E_s used.

Solid benzene at the melting point may be fitted well by the methods summarized for methane and nitrogen, but the parameters E_s and θ so obtained were found inapplicable to the solid-like structure in the liquid. Conceivably the solid-like degrees of freedom in benzene undergo a change as melting occurs. This is certainly indicated by the high entropy of fusion (8.46 e.u.) as compared with that of, say, argon (3.35 e.u.). Accordingly, the partition function was fitted to the liquid at the melting point and the values of E_s and θ were chosen to give the calculated solid-like contribution.

(3) *Partition Function including Clustered Molecules.*—Some comments may be necessary to understand equation (4). If $D = 0$, the gaslike part of the equation becomes the partition function corresponding to the monatomic gas as it should be, while if the first term inside the second curled brackets is negligible compared to the second, the expression becomes the partition function of the diatomic molecule.

A similar consideration is applied to equation (3), which includes the sum of states of an oscillator which transforms to a free rotator at higher temperatures.

Summary.—The method of significant structures applied to the liquid state is summarized. The theory is applied to methane, nitrogen, and benzene as well as to the rare gases, with good success. The numerical values of the parameters which give the best fit of the various liquids at the melting point are obtained. An attempt to fit the partition function to the critical point, by introducing diatomic clusters, is described.

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*HYPERFINE ABSORPTION SPECTRUM OF CH(COOH)$_2$**

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It has been shown elsewhere[1, 2, 3] that it is possible to produce oriented radicals with the formula $CH(COOH)_2$ in single crystals of malonic acid by subjecting these crystals to 50 kv. X-ray damage at room temperature. The orientation of the $CH(COOH)_2$ radical in the crystal lattice, and its electronic structure, was investigated by an analysis of the anisotropy of the proton[1,2] and carbon-13[3] hyperfine structure that was observed in the paramagnetic resonance at X-band (9,500 Mc, 3400 gauss) and at K-band (24,100 Mc, 8600 gauss). The proton hyperfine interaction involves the CH group proton, and is described by the spin Hamiltonian,

$$H = AS_zI_z + BS_xI_x + CS_yI_y \tag{1}$$

where $A = -29 \pm 2$ *Mc*, $B = -61 \pm 2$ *Mc* and $C = -91 \pm 2$ *Mc*. The z-axis is along the CH bond direction, and the x-axis is perpendicular to the plane of the three carbon atoms. The purpose of the present paper is to report the observation of linearly polarized radiofrequency absorption at 13.4 ± 0.2 and 15.5 ± 0.2 Mc in single crystals of X-ray damaged malonic acid in zero applied magnetic field.

The observed transitions are between the stationary eigenstates of the spin Hamiltonian (1). Let $\alpha\alpha$, $\alpha\beta$, $\beta\alpha$, $\beta\beta$ represent the electron (e) and proton (p) spin functions, $\alpha(e)\alpha(p)$, $\alpha(e)\beta(p)$, $\beta(e)\alpha(p)$, $\beta(e)\beta(p)$. The spin eigenfunctions ψ_i and eigenenergies E_i of (1) are

$$\psi_1 = 1/\sqrt{2}[\alpha\beta + \beta\alpha] \qquad E_1 = {}^1/_4[-A + B + C] \tag{2}$$

$$\psi_2 = 1/\sqrt{2}[\alpha\alpha - \beta\beta] \qquad E_2 = {}^1/_4[A - B + C] \tag{3}$$

$$\psi_3 = 1/\sqrt{2}[\alpha\alpha + \beta\beta] \qquad E_3 = {}^1/_4[A + B - C] \tag{4}$$

$$\psi_4 = 1/\sqrt{2}[\alpha\beta - \beta\alpha] \qquad E_4 = {}^1/_4[-A - B - C] \tag{5}$$

There are six allowed magnetic dipole transitions between these states and each is linearly polarized in the x, or y or z-direction, as follows.

$$\psi_1 \leftrightarrow \psi_2,\ \psi_3 \leftrightarrow \psi_4 : y\text{-polarized}$$
$$\psi_2 \leftrightarrow \psi_3,\ \psi_1 \leftrightarrow \psi_4 : z\text{-polarized}$$
$$\psi_1 \leftrightarrow \psi_3,\ \psi_2 \leftrightarrow \psi_4 : x\text{-polarized}$$

Since the levels (2)–(5) are nondegenerate for $A \neq B \neq C$, the radical has no first-order magnetic moment, and no first-order magnetic interaction with local fields in the crystal.

We have observed two of the six possible transitions. They are $\psi_1 \leftrightarrow \psi_2$, and $\psi_2 \leftrightarrow \psi_3$. A high sensitivity rf bridge of a type used for nuclear resonance experiments was employed,[4] together with an X-irradiated malonic acid single crystal sample with a volume of the order of 1 cm.^{3} The sample temperature was 77°K; no absorption could be detected at room temperature, presumably because of line broadening. Spectra were taken by tuning the bridge to various preselected frequencies and then varying a weak applied field through zero. The applied field

was modulated at 400 cps for purposes of phase sensitive detection. The y-axis polarized transition was observed at a frequency of 13.4 ± 0.2Mc. This agrees to within the experimental with the frequency of $^1/_2(A - B) = 16 \pm 3$ Mc that is predicted for the y-axis polarized transition, $\psi_1 \leftrightarrow \psi_2$. The second equally intense absorption at 15.5 ± 0.2 Mc was found to be z-axis polarized. This agrees satisfactorily with the frequency $^1/_2(B - C) = 15 \pm 3$ Mc that is predicted for the z-axis polarized transition, $\psi_2 \leftrightarrow \psi_3$. In these experiments the zero-field signal/noise ratio was *ca.* 50/1. The estimated line-width (on a frequency scale) at zero-field is of the order of one megacycle. Further work on this subject is in progress.

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DETERMINATION OF THE PARAMETERS FOR HELIX FORMATION IN POLY-γ-BENZYL-L-GLUTAMATE

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In an earlier investigation[1] it was shown that the helix-coil transition in poly-γ-benzyl-L-glutamate was dependent upon the temperature and solvent composition, while the sharpness of the transition depended upon the molecular weight. Recently there have been several more or less equivalent theoretical descriptions of the transition.[2-7] Since the dependence of the shape of the transition curve on molecular weight is crucial in the comparison of theory with experiment and in the evaluation of the theoretical parameters, we have obtained new experimental data on a new and well characterized sample of low molecular weight. In this paper we present these data and attempt a critical comparison with theory.

Theory.—We use the theory of the transition in the form proposed by Zimm and Bragg.[3] In brief, the configurations of the chains depend upon two parameters, s and σ. The parameter s is essentially the equilibrium constant for the addition of an amide residue to a section of helix, the amide residue being initially at the end of the random portion of the chain adjacent to the helical portion. The parameter σ is a factor, much less than unity, that measures the difficulty of starting a helical section; σs is the equilibrium constant for the formation of the hydrogen bond that initiates a helix. For very long chains the fraction of residues in the helical form, θ', is effectively equal to θ, the fraction of possible hydrogen bonds actually formed, and this is given by

$$\theta = d \ln \lambda / d \ln s, \tag{1}$$

where λ is the larger value of

$$\lambda = (1/2)\ \{1 + s \pm [(1 - s)^2 + 4\sigma s]^{1/2}\}. \qquad (2)$$

When the chains are short it is to be expected that there will be at the most one helical section per chain in view of the fact that σ is much smaller than unity. Then it is easy to derive a formula for θ' in straightforward fashion by summing the statistical weights of the various configurations; the result for a chain of n residues is

$$\theta' = \frac{\sigma \sum_{k=1}^{n-3} (k + 3)(n - k - 2)s^k}{n\left[1 + \sigma \sum_{k=1}^{n-3} (n - k - 2)s^k\right]}$$

$$= \frac{n(s - 1) - 2 - s^{-n+2}[3(n - 2)s^2 - (7n - 16)s + 4(n - 3)]}{n(s - 1)\ \{1 + \sigma^{-1}(s - 1)^2 s^{-n+1} - [(n - 3)(s - 1) + s]s^{-n+2}\}}. \qquad (3)$$

According to the theory, s has a critical value at unity in the neighborhood of which the transition from random to helical configuration occurs. The sharpness of the transition depends both on the value of σ and on the chain length, being sharper at smaller values of σ and at greater chain lengths. Above a certain value of the chain length, which in the present case turns out to be of the order of several hundred amide residues, the course of the transition becomes substantially independent of chain length.

In order to relate the theory to the data of the present experiments we assume that s varies with temperature but that σ does not. The justification for this assmption lies in the interpretation of σ as a measure of the configurational restrictions imposed on the first turn of the helix by the formation of the first hydrogen bond; σ is thus expected to be related to a part of the entropy of formation of the first turn of the helix and to be relatively independent of temperature. On the other hand the parameter s, which is effectively an equilibrium constant, is related to the energy of hydrogen bonding through a Boltzmann factor, and is therefore expected to be temperature dependent. In fact, the relation of s to temperature should be a thermodynamic relation of the form

$$d \ln s/dT = \Delta H/RT^2, \qquad (4)$$

where T is the absolute temperature, R the gas constant, and ΔH is the heat of adding one amide residue to a section of helix, the residue being initially in the random state.

To relate the data to the theory, we assume that σ is a constant and that $\ln s$ is proportional to $(T - T_c/)TT_c$, and vary σ and the proportionality constant until the best agreement between theory and experiment is obtained. Since with the assignment of two parameters the theory can specify the fraction of residues in the helical form as a function of temperature and of chain length, our experimental aim has been to provide such data and to see if it could be fitted by particular values of the two parameters.

Experimental Procedure.—Inasmuch as earlier work[1, 8-10] had demonstrated that the helical and coil forms of several different polypeptides have quite different and

characteristic values of specific rotation, $[\alpha]_D$, we assume that from the measurement of this quantity we can deduce directly the fraction of residues in the helical configuration, that is θ'. The validity of this assumption for short helical regions is considered in the next section.

It has already been shown that the data on the thermally induced helix-coil transition for very high molecular weight poly-γ-benzyl-L-glutamate in ethylene dichloride-dichloroacetic acid mixtures is very well fitted by the theory. Consequently, the need is for similar data, i.e., $[\alpha]_D$ as a function of temperature, for several samples of low molecular weight. It is necessary that the molecular weights of these samples be accurately known and that their molecular weight distribution be quite narrow. As the result of a previous investigation of low molecular weight polypeptides[11] two samples of poly-γ-benzyl-L-glutamate that satisfied these requirements were available. These two samples of poly-γ-benzyl-L-glutamate had been prepared by Dr. J. C. Mitchell by polymerizing the *N*-carboxy anhydride in dimethylformamide using *n*-hexylamine as initiator. Their number average molecular weights were available from the mole ratio of *N*-carboxy anhydride to initiator (A/I). Their weight average molecular weights had been determined by the Archibald approach-to-equilibrium procedure in an analytical ultracentrifuge. Sample number 19 had a number average degree of polymerization equal to 20 and a weight average equal to 26. Sample number 26 had a number average of 40 and a weight average of 46. The uncertainty in these figures is about one unit. The ratio of the weight to number averages shows that the chain length distributions are moderately narrow but not so much so as to eliminate entirely the possibility of some effect due to polydispersity.

Optical rotations were measured with a Rudolph precision spectrophotometric polarimeter, Model 80S, using a zirconium arc as light source. By means of a water-jacketed polarimeter tube and housing measurements were made in the temperature region of 7 to 50°C.

Initially measurements were made in ethylene dichloride-dichloroacetic acid (20:80) as was done previously[1] but it soon became evident that the dichloroacetic acid content would have to be reduced in order for the transition in the short chain polypeptides to appear in the available temperature range. Consequently a mixture of 30:70 by weight was adopted. A very high molecular weight sample[15] (RK-397) was also measured in this solvent mixture. It has a weight average degree of polymerization of 1500.

The two low molecular weight samples were carefully purified by repeated dissolution in dioxane and precipitation with ethanol (95%). The samples were then lyophilized from dioxane solution and vacuum dried. The concentration used in determining the specific rotation was based on the dry weight of this product.

Results and Discussion.—The comparison of the experimental results and the theoretical curves is shown in Figure 1. By plotting the optical rotation against $T - T_c$, where T_c is the temperature of the midpoint of the transition for the sample of highest molecular weight, the older and newer data can be compared on the same basis in spite of the difference in solvent composition and actual temperature. By trial of several values, we have found that the new data are best represented if the constant σ in the theory is assigned the value 2.0×10^{-4}. The selection of the proportionality factors for the relation of θ' to $[\alpha]_D$ and ln$s$ to $(T - T_c)/TT_c$ is then

fairly uncomplicated. From the latter factor we calculate, by equation (4), that ΔH is +890 cal/mol.[16] We estimate the probable error of σ as about 30 per cent, of ΔH, about 15 per cent.

While the validity of the optical rotation as a measure of the fraction of residues in the helical form now seems to be generally accepted for the case of long helices, its use in this investigation encounters the new problem of the effect of the residues at the end of short helices. In order to assess the importance of this effect, we have calculated theoretically the optical rotation of short continuous helices according to the method of Fitts and Kirkwood.[12] The latter actually carried out the calculation only for infinite helices, but they give formulas for the finite case. We adopt

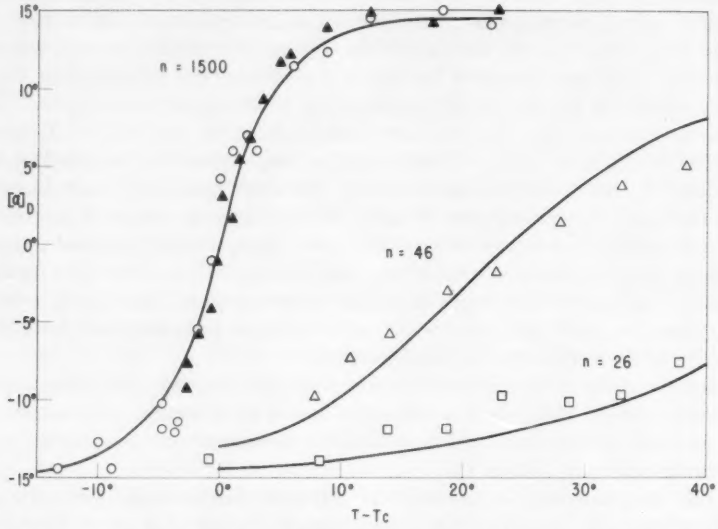

FIG. 1.—Theoretical curves of fraction of helical residues, θ', and experimental results for the optical rotation, $[\alpha]_D$, as a function of temperature, T, minus critical temperature, T_c, for samples of various degrees of polymerization, n. ○, data of ref. 1, n = 1,500; ▲, data of this work, n = 1,500; △, n = 46; □, n = 26. T_c for data of ref. 1, 28.7°C; T_c for this work, 11.8°C. Solvent in ref. 1, dichloroacetic acid, 80 parts by weight, ethylene dichloride, 20 parts; for this work, dichloroacetic acid 70 parts, ethylene dichloride 30 parts.

their model, a helix of continuous polarizability with the principal axis of the point-by-point polarizability tensor directed along the tangent to the helix at that point. Then the optical rotation per unit length of helix is proportional to the integral

$$\frac{1}{\theta_L}\int_0^{\theta_L} \frac{(\theta_L - \theta)[(\theta \sin\theta - 2)(1 - \cos\theta)]}{[2(1 - \cos\theta) + \gamma^2\theta^2]^{3/2}} \left[\cos\theta + \gamma^2 - \frac{3(\sin\theta + \gamma^2\theta)^2}{2(1 - \cos\theta) + \gamma^2\theta^2}\right] d\vartheta \tag{5}$$

where θ_L is 2π times the number of turns in the helix and γ is the ratio of the distance between the turns to the radius of the helix. The value of γ was taken to be 0.478, appropriate for the alpha helix of Pauling and Corey,[13] and the integral was evaluated by numerical integration for a series of values of θ_L. The results were: for a

helix of one-third of a complete turn, 0.10; for two-thirds turn, 0.33; for one turn, 0.45; for from one to three turns, values between 0.45 and 0.47. (Fitts and Kirkwood[12] obtained an asymptotic value of 0.492 for an infinitely long chain.) Thus the optical rotation per unit length or per residue appears to be essentially independent of length for any such helix with more than one turn.

Of course, the possibility remains that the model helix discussed above may approach unusually rapidly to the asymptotic value of the optical rotation per residue. We should therefore investigate what are the lengths of the helices that we are actually encountering in our experiments. For short chains the lengths may be calculated by a formula analogous to equation (3); the result is best expressed in terms of the average number of helical residues in all chains that contain any helical residues at all. For $n = 26$ we find, using a value of 2×10^{-4} for σ, that this average number ranges from 11.3 at s equal to unity to 18.7 at s equal to 1.25. Since the mean number of helical residues in all chains is quite small in the above cases (see Fig. 1), it is clear that most of the chains at values of s above unity contain either no helical residues or a substantial number, but never a small number; this is indeed a consequence that might have been anticipated from the small value of the initiation parameter, σ.

With 3.6 residues per turn, we conclude that in the sample of n equal to 26 practically all the helices of interest to us contain several turns. Therefore, unless the simplified model calculation of the optical rotation described above is singularly misleading, it would seem safe to assume that the optical rotation is a linear function of the fraction of helical residues.

The same conclusion holds also for the sample of high molecular weight. An elementary calculation shows that the mean length of the helical sections of a very long chain is $1/(1 - s)$ residues when s is less than one, and certainly the mean length of the helical sections must increase as s increases. A glance at Figure 1 shows that we are not interested in values of s less than 0.9; therefore the mean length of the helical sections in the long chains is at least ten residues.

Having thus disposed of one possible difficulty in the interpretation of the optical rotation, we now turn to possible effects of the simplifications inherent in the theory of the transition. In the first place, we have assumed that σ is independent of temperature. As we have seen, σ is a measure of the extra free energy lost when three amide residues are reorganized from the random state to start a helix in comparison to the free energy lost when one segment in the random condition is added to a section of preexisting helix. In assuming that σ is independent of temperature, we are in effect assuming that the extra free energy has an entropy but no internal energy component. Since the two bonds adjoining the alpha carbon of each residue are believed to be the only ones in the chain backbone that have any freedom to twist, the entropy component of σ should represent the restriction that four of these bonds experience when entering the helix. With $\sigma = 2 \times 10^{-4}$, this represents a restriction in the rotational space of each bond by about one factor of ten, a value reasonable enough in the light of our present imperfect knowledge.

On the other hand, even if the whole extra free energy of starting a helix is supposed to be internal energy, the value of this could be no more than $kT \ln \sigma$, or about 5 kcal/mol in the present case. The resulting change in σ over the range of 50° encountered in our measurements would be only about 15 per cent, which is less

than the precision of the curve fitting process. Therefore we do not see how the assumption that σ is independent of temperature could be the source of any considerable error.

Thus the interpretation of the new data by the theory appears to be encouragingly successful. However, further studies would be most desirable, particularly with samples of short chain length in various solvents. These would be especially helpful in establishing the constancy of σ, and in elucidating the changes of s with solvent and temperature.

The quantitative improvement in the treatment of the helix-coil transition should eventually provide greater insight into the stability of protein configurations and protein denaturation. As an example, the view that globular proteins consist of helical regions interspersed with non-helical, and non-periodically organized, regions[14] can now be examined somewhat more quantitatively. While the heat content change on unfolding the helical form in aqueous solution probably ranges from positive values for some residues to minus one or two kilocalories for others,[17] a value of 1 kilocalorie is probably a fair average value for those regions of the protein that are helical. Since this is of the same magnitude as, though opposite in sign to, that found in this study, one might expect that the temperature dependence of the fraction of residues unfolded in a protein would be given by a curve similar in appearance to those in Figure 1. However the helical regions in globular proteins must be quite short, being limited at least by the dimensions of the globular molecule if not otherwise. And *independent* short helices show very broad transitions, as in Figure 1, in contrast to the almost catastrophic transitions frequently seen in the denaturation of proteins.

We are thus faced with a dilemma; the helix-coil transition offers a reasonable explanation of the optical rotatory changes accompanying protein denaturation, but it does not seem to show the sharp temperature dependence. The resolution of this problem probably is connected with the word *independent* emphasized above, i.e., if the transitions of the various helical parts of the same protein molecule are coupled in some way, the interactions should sharpen the transition. Such coupling could, for example, be mediated by disulfide bridges between parts of the molecule, which, by mechanical constraint, would force two or more otherwise separate parts of the chain to undergo the transition simultaneously. This increase in the cooperative interaction in the molecule should act to sharpen the transition.

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[16] An earlier estimate, based on fewer data, was 990 cal/mol. (ref. 3).

[17] J. A. Schellman has obtained a value of 1.5 kcal. from several lines of argument (ref. 2).

ON THE TOPOLOGY OF THE GENETIC FINE STRUCTURE

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From the classical researches of Morgan and his school,[1] the chromosome is known as a linear arrangement of hereditary elements, the "genes." These elements must have an internal structure of their own. At this finer level, within the "gene" the question arises again: what is the arrangement of the *sub*-elements? Specifically, are *they* linked together in a linear order analogous to the higher level of integration of the genes in the chromosome?

Until recently, the sensitivity of genetic analysis has been insufficient to answer this question. Mapping of a genetic structure is done by observing the recombination of its parts, and recombination involving parts of the structure that are very close together is a rare event. Observation of such rare events requires very many offspring and a selective trick for detecting the few individuals in which the event is recorded. It is for this reason that microorganism are the material of choice for studies of genetic fine structure, and have made it feasible to extend the fineness of genetic mapping by orders of magnitude. In favorable systems, the attainable resolution reaches the level of the molecular subunits of the hereditary material. and experimental testing of the linear arrangement of the finest structural details is therefore possible.

A number of cases have been investigated on this level.[2] As a rule, closely linked mutations affecting the same characteristic can be seriated in an unambiguous way, suggesting a linear model. However, the "distances" (i.e., recombination frequencies) between mutations are not always strictly additive, and certain complexities ("negative interference" effects[3, 4]) make quantitative analysis difficult. As pointed out by Muller[5] in regard to similar difficulties encountered in mapping on the chromosomal level, strict additivity of "distances" should not be taken as the criterion for the linear character of an array. A crucial examination of the question should be made from the point of view of *topology*, since it is a matter of how the parts of the structure are *connected* to each other, rather than of the distances between them. Experiments to explore the topology should ask *qualitative* questions (e.g., do two parts of the structure touch each other or not?) rather than *quantitative* ones (how far apart are they?).

In what follows, such an investigation is attempted for a small portion of the genetic structure of a virus, the "*r*II" region of phage T4. Using only qualitative tests, an examination is made of the topology of this structure at the molecular level.

The Material and the Method.—The methods for studying *r*II mutants of phage T4 have been described in detail elsewhere.[6, 7] Briefly, T4 phage of the "standard" form can multiply normally in either of two bacterial host strains, B or K. From the standard form of T4, *r*II mutants occasionally arise (detectable by their plaque morphology on B) that are defective in growth on K. An *r*II mutant *can* grow normally on K if the cell is simultaneously infected with a particle of the standard type phage. Thus, the standard type is able to perform some necessary function which the mutant cannot. Our interest is in the genetic structure that controls this particular function of the phage. The controlling structure has been traced to a small portion of the genetic map of the phage, the "*r*II" region, and various *r*II mutants can be shown to contain different blemished versions of the structure, as distinguished by certain criteria.

One criterion is the recombination test, in which two mutants are allowed to multiply within the same host cell, thereby providing an opportunity for the production of progeny which obtain parts of their genetic information from each parent. By *recombination* of the unblemished portions of the two mutant versions, some standard (i.e., non-mutant) individuals may be regenerated. To perform the recombination test with *r*II mutants, one can infect cells of strain B (in which the mutants are able to multiply) with the two mutants in question and examine the progeny for the appearance of standard type individuals by plating on cells of strain K, on which only the standard type can grow. Such recombinants will occur only if the two mutants do not contain blemishes affecting the same part of the structure. The sensitivity of the test is sufficient to resolve mutations separated by the smallest conceivable distance, i.e., that corresponding to the spacing between nucleotides in the DNA.[6]

Thus, a simple experiment gives a qualitative yes or no answer to the question of whether or not two mutations overlap. This provides a key to the study of the topology of the structure.

The postulates upon which the procedure is based may be stated as follows:

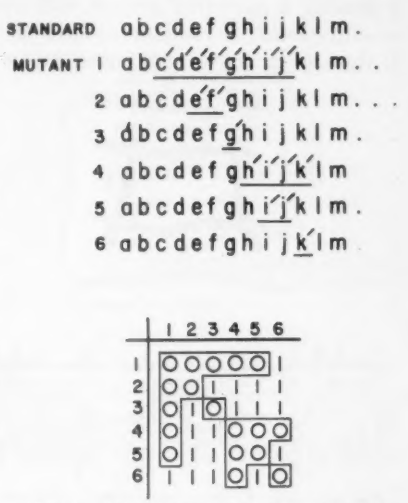
(1) A hereditary structure in the phage determines its activity (i.e., ability to multiply in K). *The standard structure consists of a set of elements, alteration of one or more of which produces a mutant (inactive) form.* The subset of altered elements in any particular mutant is assumed to be continuous and not to enclose an unaltered region within its boundaries.

(2) When two or more phage particles infect the same cell, *genetic recombination* may occur: *each element of the hereditary structure in a progeny phage particle is derived from the corresponding element in one or the other of the parental phages.* The nature of the recombination mechanism, (e.g., whether involving material transfer of parental parts or a partial copying mechanism) is immaterial.

Given these postulates, the recombination test, applied to two *r*II mutants, reveals whether their mutations overlap or do not. By applying this test to many mutants. it should be possible to determine the manner in which the various parts or the structure are interconnected.

Effect of the topological nature of the structure: A few illustrations for model structures of different sorts may be helpful in clarifying this approach. Consider an example of an unconnected structure: a pack of cards. A complete pack of 52 subunits constitutes an active (standard) structure. The individual cards are interrelated as members of a set, but are not connected in any fixed order. A simple alteration, such as the damage or loss of one of the cards, renders the pack inactive (mutant). However, given *two* mutant packs, it is possible to produce a standard pack by recombination, *but only if the two mutations do not intersect*, i.e., the same card must not be altered in both cases. If such a test were extended to an unlimited number of mutant packs, comparing them two-by-two and in each case recording only whether a good pack *could* or *could not* be produced, the mutants would fall into fifty-two categories. Thus, the data would reveal that the standard structure consists of fifty-two distinct parts and there would be no indication of any connections between the parts.

Consider now a connected standard structure of linear topology without branches or loops: a perfect tape recording of a piece of music. Such a structure can be rendered unacceptable by a blemish—one false note, perhaps, or a blank interval (due to a jump of the tape, say). Given two independent "mutant" versions, it may be possible to fabricate a standard one by recombination, but only if the two blemishes do not overlap. The exact mechanism is immaterial—either reciprocal exchange of parts (scissors and paste) or copy-choice (partial playback) will do. Now if various mutant versions are tried, two-by-two, in each case noting only whether or not successful recombination is possible, a new sort of result may be found with this connected structure that was not possible with an unconnected pack of cards. A blemish in the recording can involve a *segment* of the structure (and still be "simple" according to the restriction in the first postulate). It may therefore occur that one mutation intersects two others that do not themselves intersect. Given enough defective versions, the yes-or-no results of recombination experiments would enable one to construct a linear map showing the various defects in their relative positions within the standard structure.


STANDARD a b c d e f g h i j k l m .
MUTANT 1 a b c d e f g h i j k l m . .
2 a b c d e f g h i j k l m . . .
3 a b c d e f g h i j k l m .
4 a b c d e f g h i j k l m
5 a b c d e f g h i j k l m .
6 a b c d e f g h i j k l m
1 2 3 4 5 6
1
2
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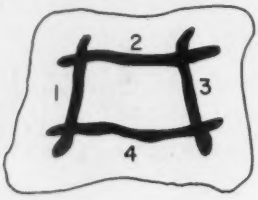
5
6

FIG. 1.—(*a*) A linear standard structure is indicated as a series of elements. Alteration of any element or continuous group of elements produces a "mutant" structure. Six examples are given, arranged in dictionary order.

(*b*) The recombination matrix for these six mutants. A one indicates that standard type can be produced by recombination, a zero that it cannot.

A linear topology places certain restrictions on the pattern of results. Representing the standard structure as a sequence of elements *a b c d* . . ., the structure becomes mutant upon alteration of one element or a continuous series of them.

A collection of such mutants can be arranged in *dictionary order* according to the first altered element, as in Figure 1a. If the six mutants shown are *crossed* (i.e., subjected to the recombination test) in all possible pairs, the pattern shown in Figure 1b is obtained. A zero indicates that standard type cannot arise by recombination, while a one indicates that it can. Diagonal values (i.e., for crosses of mutants with themselves) are, of course, always zero, and non-diagonal values are zero or one according to whether or not the two mutations in question overlap. The matrix has a characteristic feature which is a necessary consequence of the fact that the mutants have been arranged in dictionary order. By starting from a point on the diagonal, and moving to the right (or down), one reads off the results of crossing a particular mutant with successive ones on the list. Once the *first* non-intersecting mutant is reached, there must be *no further* intersecting ones. (If there were, they would belong higher up on the list.) Therefore, the zeros in any row or column (starting from the diagonal and moving to the right or down) must form a series unbroken by ones.



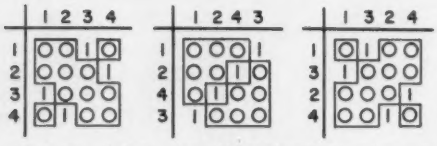


FIG. 2.—(*a*) A two-dimensional structure is shown, with four "mutations" mapped on it.
(*b*) The three possible arrangements of the recombination matrix for these "mutants."

Conversely, given the data obtained on a set of mutants, *if the mutants can be listed in an order such that the matrix satisfies this criterion, their mutations can be represented as segments of a simple linear structure.*

This is by no means possible for any set of data. A two-dimensional structure, for example, can give quite different results. In two dimensions it would be possible for four mutations to intersect as shown in Figure 2, where mutation 4 intersects with 1 and 3 *without* intersecting with 2. Such a situation would be impossible in a linear structure. Pairwise crosses of the four mutants would give the results shown. By listing the mutants in various ways, three different patterns may be obtained, but in no case is it possible to persuade all the zeros to form uninterrupted rows and columns.

And so on for three dimensions, branched structures, etc., each of which, if explored with a sufficient number of mutants, would give characteristic results impossible to represent in one dimension. For example, if crosses of six mutants were to give the results shown in Figure 3b, a branched structure as in Figure 3a would be required to account for them. Note that a single occurrence of a one where a zero should be is sufficient to force this conclusion. It would be an interesting mathematical problem to derive the characteristic feature of the matrix for each kind of topological space.

Experimental.—Our objective is to examine the topology of the structure in phage T4 that controls its ability to multiply in K, and specifically to make a rigorous test of the notion that the structure is linear.

The experiment consists of starting with a single particle of standard type T4 phage and isolating from it many independent, spontaneously-arising *r*II mutants, each of which may contain an alteration in a different part of the structure. These mutants are to be crossed with each other, noting in each case whether standard type recombinants do or do not appear. It is then to be determined what sort of structure is required to account for the results.

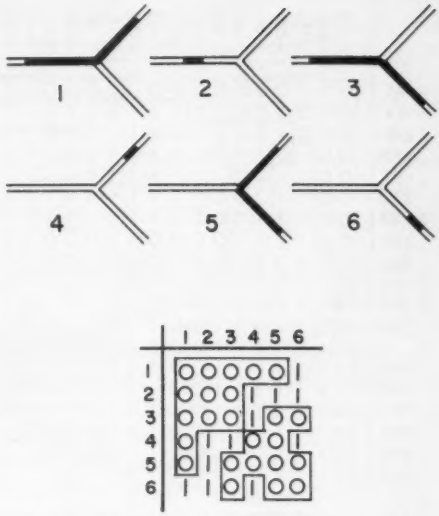


FIG. 3.—(*a*) Six possible "mutations" are shown in a branched structure. (*b*) A recombination matrix for these six "mutants."

Choice of non-reverting mutants: Most *r*II mutants have some tendency to revert spontaneously to standard type. This is a serious limitation since, when such a mutant is used in a cross, one cannot readily distinguish standard type particles arising by recombination from those due to reversion. To circumvent this difficulty in the present experiment, only those mutants are used *for which reversion has not been detected.* About one in fifteen spontaneous *r*II mutants is extremely stable against reversion. The reversion test is most stringent, since revertants would be readily detectable on K in a proportion as low as 10^{-8}.

Another advantage in choosing non-reverting mutants is that they are, *a priori*, most likely to include the ones in which *large* alterations have occurred, thereby giving a set rich in intersections.

The 145 non-reverting *r*II mutants used in the present study were chosen by screening some 2,000 spontaneous *r* mutants[8] of T4 and rejecting any that were not of the *r*II type, or were seriously "leaky" (i.e., able to grow partially on K), or reverted detectably. The chosen mutants were crossed in many pairs, in each case testing for the appearance of standard

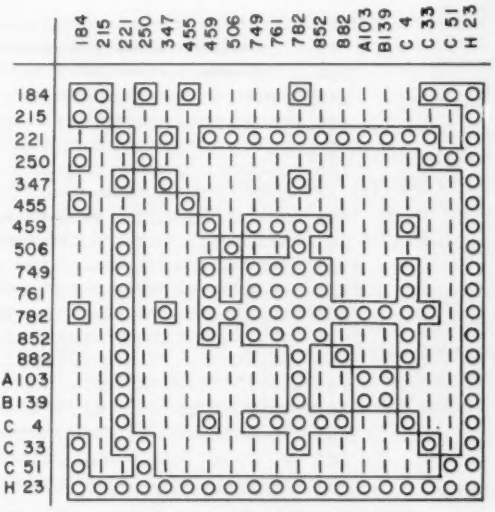


FIG. 4.—Recombination matrix for 19 *r*II mutants of phage T4, arranged in arbitrary order.

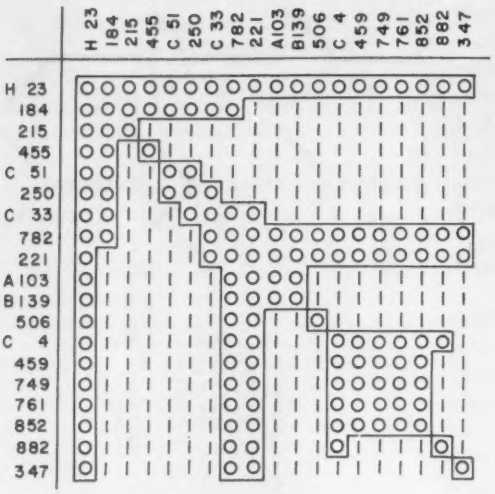


FIG. 5.—Recombination matrix for the mutants of Figure 4, rearranged in "dictionary order."

type progeny. With the procedure used (a simple spot test[7]), the result *zero* for a cross indicates that, among the progeny from cells infected with the two mutants in question, standard type particles are fewer than one in about 10^4.

Results: Figure 4 gives the results for a family of nineteen mutants related to each other by intersections. These have been crossed in all possible pairs and are listed in the order in which the mutants were isolated.

Are these data consistent with a linear structure? This can be decided by attempting to rearrange the mutants in an order which brings the zeros together into unbroken series. At first glance this might appear to be a formidable undertaking (the number of permutations of 19 mutants is greater than 10^{17}), but in practice it can be readily accomplished. In Figure 5 the mutants have been successfully rearranged. Thus, all the mutations can be represented as portions of a linear space, as shown in Figure 6

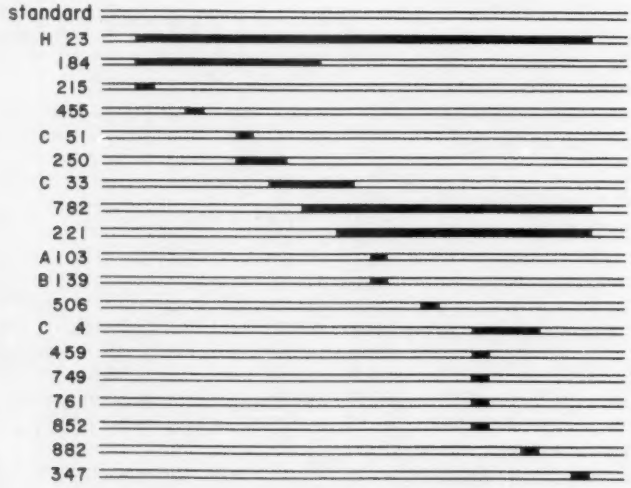


FIG. 6.—Relative positions of the mutations, as deduced from the data of Figure 5.

These results *rule out* an unconnected (pack of cards) structure, *are compatible with* a linear structure, and *do not require* any higher order of dimensionality.

The order shown in Figure 6 is not the only possible satisfactory one, since certain groups can be freely permuted without making any difference (e.g., *r*215. *r*184, *r*C51, *r*250, *r*C33, *r*782, *r*221, and *r*347). The number of uniquely ordered individuals increases as more mutants are brought into play.

In Figure 7 data are given for 145 non-reverting mutants, the mutants having been arranged in a satisfactory order, although not all the possible pairs have been crossed. All the results can be represented on a map in one dimension, as shown in Figure 8.

Functional units: The recombination test has shown that the hereditary structure controlling the ability of the phage to multiply in K consists of many parts. While alteration of any one of these leads to the same apparent physiological defect, it does not necessarily follow that all of the *r*II mutations block the same unitary function. For instance, growth in K could require a series of reactions, absence of any one of which would suffice to block the end result. It is therefore of interest to see whether the *r*II region can be subdivided in a functional sense.

It will be recalled that the needed function can be supplied to a mutant by simultaneous infection of the cell with standard type phage. Suppose that K is infected with two mutants simultaneously, as illustrated in Figure 9 (*trans* arrangement). If the two defects concern independent functional processes, the system should be active, since each mutant is intact with respect to the function affected in the other. However, if both mutants are defective in the same unitary function, the combination should still be inactive.

A proper control for this experiment is provided by testing the same genetic material in the *cis* arrangement (Fig. 9), i.e., with both mutations in one of the phages, the second phage being standard type. (In order to perform this test, the double mutant must be previously obtained by recombination.) The physiological activity of the *trans* arrangement is to be compared with that of the *cis*. If the *trans* is relatively inactive, a functional affiliation is indicated.

As applied to *r*II mutants, the *cis* test has invariably given an *active* system, as is to be expected when the standard type is present. The *trans* test divides the mutants into two clear-cut groups. Any mutant of group A can complement any one of group B, giving activity similar to that obtained in the *cis* configuration. Among mutants of the same group, however, the *trans* configuration is essentially inactive. Thus, the mutations can affect either of two distinct functional units, as defined by the *cis-trans* test. A functional unit so defined has been called[5] a "cistron."

When two mutants affected in different cistrons are tested for recombination by the usual spot test, their complementary action in lysing K leads to a massive response qualitatively different from that obtained due to recombination between mutants of the same cistron.[7] Such a massive response is indicated in Figure 7 by a two. A few *r*II mutants are defective in *both* functions, that is to say, complement *neither* A *nor* B group mutants, and therefore have defects affecting *both* cistrons.

It is important to point out that functional complementation, when it occurs, is not due to the formation of standard type recombinants inside K; full comple-

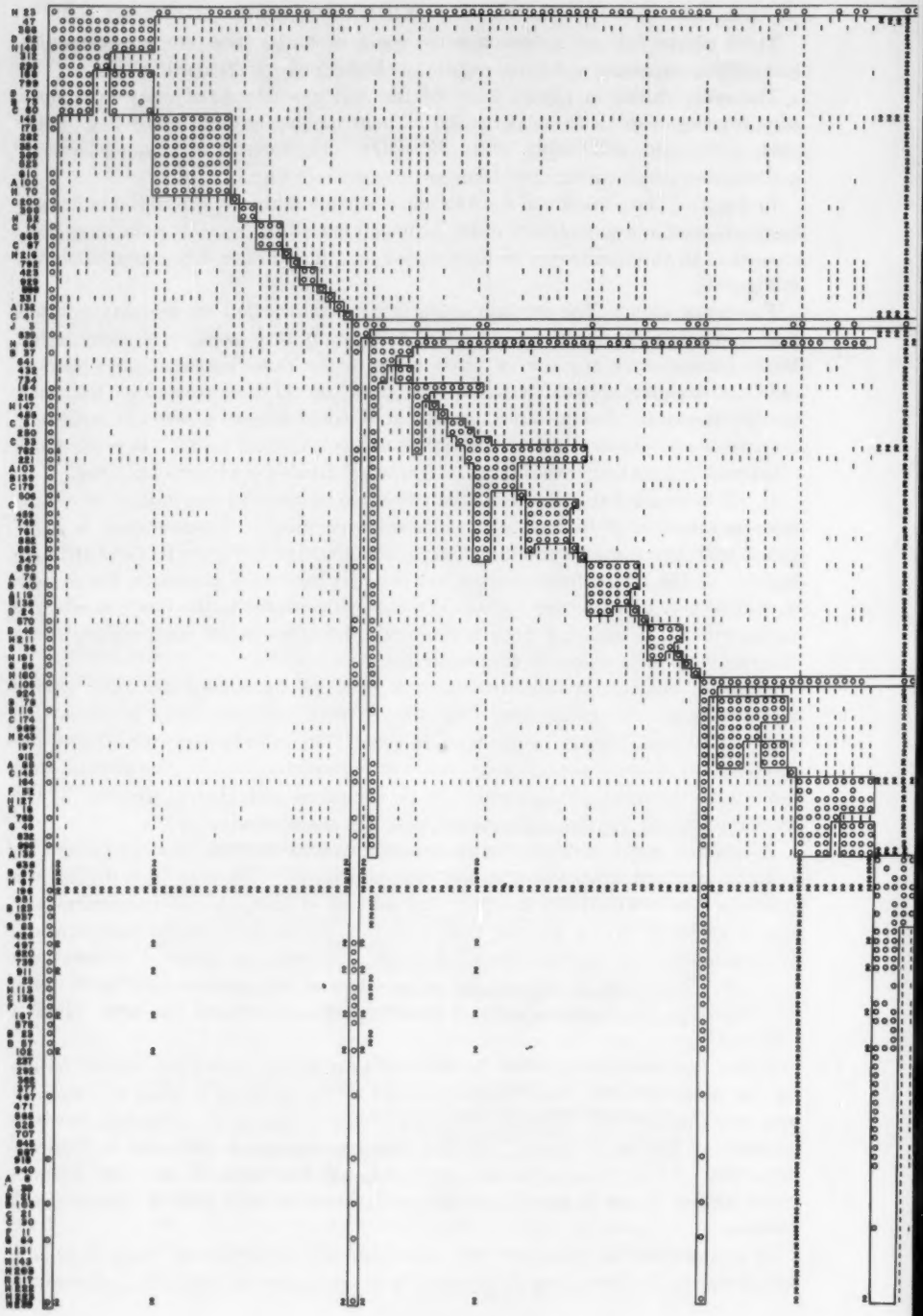


FIG. 7.—Recombination matrix for 145 *r*II mutants of phage T4. A two indicates a massive response, as occurs in crosses between mutants affected in different cistrons.

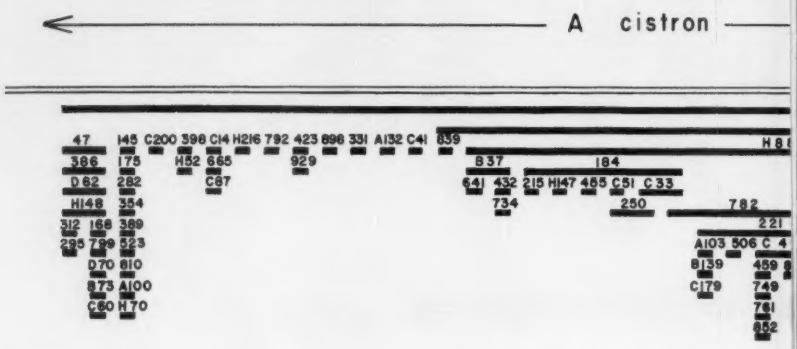


FIG. 8.—A genetic map deduced from the recombination data of Figure 7. Each cistron represents a separate functional unit as defined by the *cis-trans* test.

mentation may occur even with mutants giving very little recombination. Thus, if K is mixedly infected with *r*164 and *r*638, whose mutations lie very close together, the cells lyse nicely even though very few of them liberate any standard type particles. Conversely, if K is infected with *r*164 and *r*168, practically none of the cells lyse, even though these mutants are capable (in growth on B) of producing standard type recombinants in most of the mixedly infected cells.

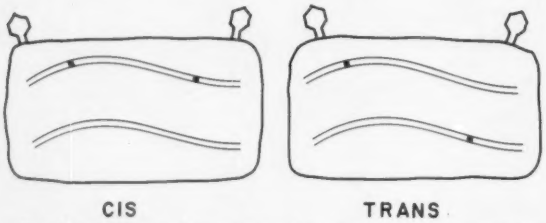
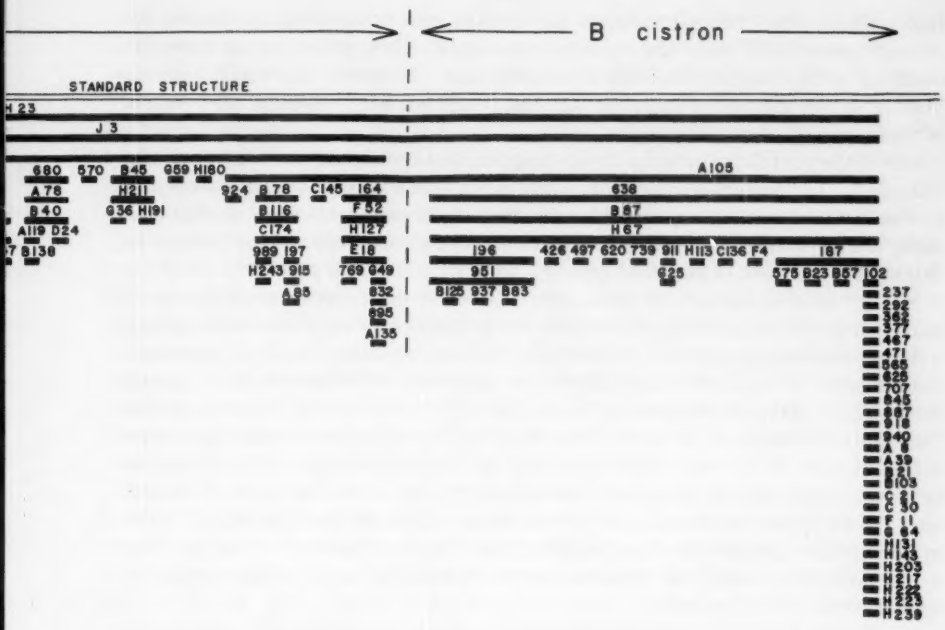


FIG. 9.—A schematic representation of the *cis-trans* comparison for testing the functional affiliation of two mutations. In the *trans* case each infecting phage carries a single mutation while in the *cis* one of the phages carries both.

Correlation of recombination and function tests: How does the assignment of mutations into cistrons by the *functional* test correlate with their locations on the map derived from the independent *recombination* test? It turns out that the map may be split into two portions by the divide indicated in Figure 8 as a vertical broken line. All mutants assigned to group A by the functional test have mutations located to the *left* of the divide on the basis of recombination. All mutations causing functional defect B lie to the *right* of the divide. Those few mutants which are defective in both functions have mutations extending to both sides.



Thus, each cistron corresponds to a sharply limited segment of the linear structure.

Discussion.—In genetic mapping, mutation and recombination are both necessary, but emphasis can be placed upon one or the other. The conventional map is made by using mutations merely as "markers," the structure being surveyed by measurements of the frequency of recombination between markers. The mapping method we have used here is a complementary one, in which the frequency of recombination is unimportant, but, in effect, mutations have been used to explore the structure.

The particular mutants used in the present work represent a special class of the spontaneously-arising *r*II mutants, having been chosen for their non-reverting and non-leaky character. Mutants containing large structural alterations would naturally be found in this group, and such large mutations are necessary for overlaps of the sort needed to test the topology of the structure. In fact, *r*II mutants that show any tendency to revert behave as though their mutations are localized to points and would have been useless in this approach. The omission of leaky mutants is perhaps part of the reason why partial complementation effects did not cause difficulty in the assignment of the mutants to clean-cut cistronic groups. This difficulty, which has occurred in some other systems,[2] has also been encountered with some leaky *r*II mutants.

The postulates on which the analysis is based are, of course, idealizations when applied to an experimental system. The standard structure is not necessarily unique. There are many examples in genetics where the observable effect of a mutation may be compensated for by a "suppressor" mutation at a different loca-

tion. Thus, when two *r*II mutants are crossed, the double mutant formed by recombination could have the standard phenotype, thereby causing an apparent doubling of the number of standard recombinants. However, this would have no bearing on the method of mapping that has been used here, which is independent of the frequency of recombination.

One of the postulates includes the assumption that each mutation is topologically "simple." In a linear structure this would mean that a mutant should not contain two or more alterations separated by an unchanged segment. Such double mutants are, of course, possible, but were rare enough not to cause complications in this study. A stock of standard type T4 typically contains a proportion of about 2×10^{-4} of spontaneous *r*II mutants. Therefore, on the assumption that each mutation occurs as an independent event, the proportion of the mutants containing a second mutation would be expected to be of the order of one in several thousand. Such double *r*II mutants would almost certainly not revert detectably. Among the roughly 2,000 mutants screened to select the 145 non-reverting mutants used in this study, one instance of a double mutant was in fact detected having a point defect in each of the two cistrons. When this mutant was crossed to standard type, two single mutant segregants were obtained, an A cistron one and a B cistron one. (This result does not occur for true large mutations, such as *r*H23.) The mutant (*r*928) has therefore been omitted from Figures 7 and 8. Note that the use of mutagens to raise the mutation rate is undesirable, as it would increase the proportion of double mutants.

Has the foregoing test of a one-dimensional scheme been sufficiently exhaustive? Even a set of nonsense data might happen to be compatible with a linear representation. Suppose one were to construct a matrix by tossing a coin to determine whether each "cross" gives zero or one. What is the probability that the "mutants" of such a random pattern would satisfy the criterion for a linear topology? For n "mutants," the number of matrix elements to be determined is $n\ (n-1)/2$. Each element is either zero or one, so there exist $2^{n(n-1)/2}$ different patterns, all equally probable. Some patterns will fit the criterion for linearity, namely those in which the zeros in each row form an unbroken series, starting from the diagonal. The first row, for instance, will be satisfactory if it contains no non-diagonal zeros, or a zero adjacent to the diagonal, or two zeros adjacent to the diagonal, etc. Thus, the first row can be satisfactory in n ways, the second row in $n-1$ ways, and so forth. Altogether, there are $n!$ satisfactory patterns, and the probability of obtaining one of these at the first go is $n!/2^{n(n-1)/2}$.

It may be, however, that an unsatisfactory pattern can be converted into a satisfactory one by rearrangement of the rows (and columns), and any given set of data may be subjected to $n!$ such arrangements. Although many of these will give redundant patterns, multiplying by this factor gives a *maximum* estimate for the chance that random data would be compatible with a linear scheme.

For $n = 19$, as for the data of Figure 4, this maximum estimate is approximately 10^{-17}. Considering the much larger set of data for Figure 7, the possibility of a fortuitous fit with a linear topology all but vanishes.

Therefore, the observed data are manifestly not random. However, this estimate does not really apply with the same force to the question of whether the structure is linear. The problem really is: given a topological space which is other than

linear, what is the probability that this will not make itself felt (in terms of exceptions) when a given number of mutants is studied? The answer depends upon the assumptions made as to the nature of the space and the distribution of mutations within it. In the case presented above, the assumption that the matrix elements are random amounts to assuming a very complex space indeed and a distribution of mutations such that intersections are very common. This leads to a particularly low probability that the results will be compatible with a linear order.

Of particular interest is the question of branches. No case was experimentally observed of six mutants with the relationship shown in Figure 3b, which would require a branched map. However, it has been assumed that mutations have no effect upon the unaltered parts of the structure. Suppose instead that a loss along the main line which includes a branch point also necessarily leads to the loss of the whole branch. This means that in Figure 3a loss No. 1 is impossible (if the lower right arm is taken to be the branch). The remaining mutants can then be arranged in a simple line, in the order 3, 2, 5, 6, 4. More generally, if *many* deficiencies relating to the branch were available, one would find an apparent linear structure all along this line, with a section along the line (the portion which in reality is the branch) which is well ordered inside, but has *no* deletions reaching into it from the outside and stopping somewhere inside. Therefore, to eliminate the possibility that a given segment constitutes such a branch, it must be subdivided by a mutation reaching into it. This occurs for two mutants with respect to the A cistron (J3 and Al05), but there are none that have this property with respect to the B cistron. While this can hardly be taken as suggesting that the B cistron is a branch, it is also true that a more exhaustive study will be necessary to rule out such a possibility.[9]

It is in the nature of the present analysis that the existence of complex situations cannot be disproved. However, the fact of the matter is that a simple linear model suffices to account for the data.

In confining this investigation to *r*II mutants of T4, attention has been focused upon a tiny bit of hereditary material constituting only a few per cent of the genetic structure of a virus and representing altogether some thousand nucleotide links in a DNA chain.[6] It would seem, therefore, that the fine structure of the hereditary material, even down to its smallest molecular components, may indeed be analogous to the linear order in which the genes are integrated in the chromosome.

Summary.—The topology of the fine structure of a region of the genetic map of phage T4 is investigated by determining whether various mutations do or do not overlap. The results permit representation of the mutations as alterations in a linear structure in which the functional units defined by the *cis-trans* test correspond to unique segments. The possibility of branches within the structure is not necessarily excluded.

I am indebted to Mrs. Marion Sjodin for assistance in the isolation of many of the mutants, to Dr. Leslie Orgel of Cambridge University for suggesting the "dictionary order" analogy, and to Dr. Max Delbrück for suggestions regarding the possibility of branches and for his usual moderating influence. This work was assisted by grants from the National Science Foundation and the National Institutes of Health.

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[6] Benzer, S., these PROCEEDINGS, **41**, 344–354 (1955).

[7] Benzer, S., "The Elementary Units of Heredity," in *The Chemical Basis of Heredity*, ed. W. D. McElroy and B. Glass, (Baltimore: The Johns Hopkins Press, 1957).

[8] Mutants herein designated with a simple arabic number were derived directly from the T4 B standard type strain. Some mutants, obtained in connection with another study, were not derived directly from the original standard type, but from revertants of various (revertible) *r*II mutants, and are designated with a roman letter prefix.

[9] *Note added in proof:* Further studies of still more mutants have now turned up three instances of partial penetration into the B cistron, as well as three more cases for the A cistron.

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*MUTATION INDUCTION AND EXPRESSION IN BACTERIA**

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Demerec[1] in 1946 first demonstrated that a period of time elapses between application of mutagenic treatment and the phenotypic expression of mutations induced by the treatment. Two hypotheses have developed to account for this delay. According to the first, a mutation is induced in the gene immediately on application of the mutagenic agent to that gene; and the lag occurring between application and phenotypic expression of the mutation represents that time period required for synthesis of the altered enzyme which is under the control of the mutated gene. The second hypothesis holds that induction of mutation is not immediate after application of the mutagenic agent, and that certain chemical events or cellular synthetic processes are necessary for establishment of the mutation in the genetic apparatus. According to this theory, the delay observed is the time required for these processes to take place.

It is hardly necessary to point out that these two theories are not mutually exclusive. It is readily apparent that some finite period of time will be required for synthesis of an altered enzyme following a genetic change before any manifestation of the change can become apparent. Until recently it has not been so clear that a period of time is also necessary for establishment of the mutation within the genome.

Witkin[2] has cited considerable evidence that "ultraviolet induced mutations are irreversibly established only after an appreciable delay." She has also given evidence that the induction process is complete prior to the first postirradiation cell division and indicating that the induction process is dependent on the availability of amino acids in the incubation medium during the first postirradiation hour. Haas and Doudney[3] and Doudney and Haas[4] have presented evidence which sug-

gests that nucleic acid precursors altered *in vivo* by ultraviolet light (UV) serve as intermediates in the induction of mutation. Subsequent experiments demonstrate that "fixation" of mutation is correlated with ribonucleic acid (RNA) synthesis in the cell, and indicate that all mutations are "fixed" *before* any measureable deoxyribeonucleic acid (DNA) synthesis occurs.[5] These findings have led to the hypothesis that RNA and protein syntheses are intimately involved in replication of genetic DNA; and RNA, modified by incorporation of radiation-altered precursors, in some manner leads to a corresponding modification in the newly formed genetic DNA.

The foregoing hypothesis for UV-induced mutation implies that the mutation appears initially in the first daughter genetic DNA synthesized after irradiation. This implies in turn that a period of time is necessary between mutagenic treatment and establishment of a mutation in the genome. It then becomes of primary interest to determine at what point in the sequence of macromolecular synthetic events following UV exposure the induced mutation is capable of being phenotypically expressed. Establishment of the point at which genetic control of enzyme synthesis is initiated may clarify not only the processes involved in gene synthesis and mutation induction, but also the genetic control of cellular enzymatic activity.

Many of the aforementioned experiments on UV-induced mutation were carried out using *Escherichia coli* strain WP2 (Witkin[2]). This is a tryptophan-requiring strain, and the mutation studied was reversion to the prototrophic state (non-tryptophan-requiring). It became quite evident in these experiments that if, during the earlier postirradiation stages of incubation, the bacteria were plated on minimal medium rather than on a nutrient broth supplemented medium, a much lower level of mutation induction was obtained. This was the case regardless of the postirradiation treatment prior to plating. It occurred to us that this lower level of mutation induction might be due to inability of the induced reversions (in biochemically deficient strains) to be expressed phenotypically without some initial supplementation. In such a case, the behavior described would indicate that mutation expression is dependent on macromolecular synthetic events *following* those responsible for establishment of the mutation in the genome. This paper reports evidence suggesting that mutation expression is attained subsequent to the synthesis of DNA following UV exposure, and that it is dependent on protein synthesis following the initial synthesis of DNA.

Materials and Methods.—The mutations studied result in reversion of *E. coli* strain WP2 to the non-tryptophan-requiring state. In these experiments two plating media were used, either minimal medium or minimal medium supplemented with 2.5 per cent Difco nutrient broth. The low amino acid concentration in the latter medium allows a limited degree of mutation induction, while the tryptophan present allows unreverted cells to make enough divisions to develop small but visible colonies and also enables the reverted cells to become expressed phenotypically. The basic procedures followed for culture growth, cell synchronization, radiation exposure, culture treatment and plating, and for RNA, DNA and protein determinations have been previously described.[3–5] UV irradiation was for 10 seconds at a distance of 30 cm (964 ergs/mm^2). Unirradiated suspensions contained about 6×10^8 colony-forming cells per ml, and the irradiated suspensions

contained about 4×10^8 colony-forming cells per ml. The usual precautions to prevent photoreactivation were observed.

Results.—A correlation between RNA synthesis and "mutation fixation" in UV-irradiated cultures has previously been described.[5] The time required for mutation fixation can be quantitatively measured by short-term challenge with chloramphenicol or other agents or conditions which interfere with protein synthesis.[5] This challenge demonstrates the fraction of "potential" induced mutations "fixed" at any given interval following UV exposure. Following the challenge period, which is carried out in liquid medium, plating is on minimal agar medium supplemented with 2.5 per cent nutrient broth. This contains sufficient tryptophan for the protein synthesis necessary for mutation expression. A typical experiment relating mutation fixation (as determined by chloramphenicol challenge) to RNA synthesis is shown in Figure 1. Extensive analyses of this type have led us to the

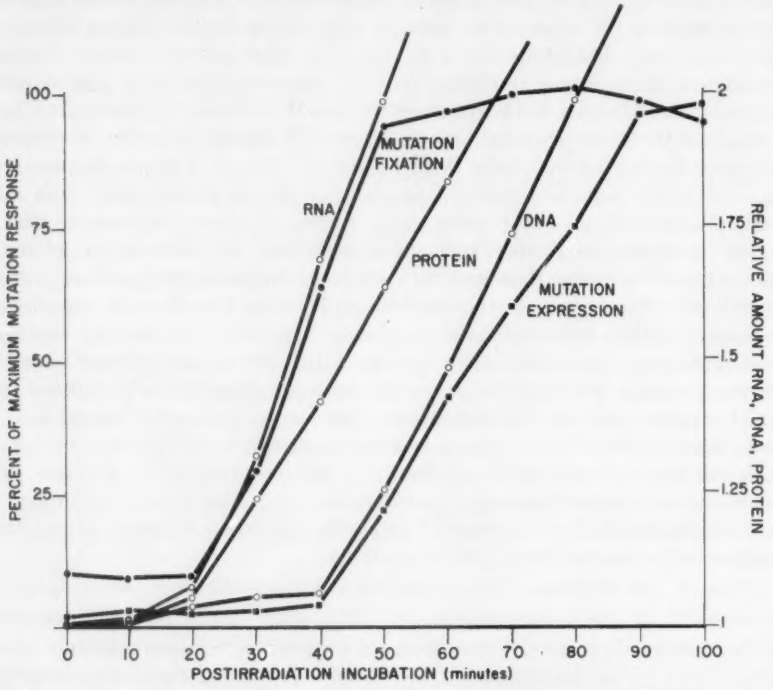

FIG. 1.—Relation of "Mutation Fixation" and "Mutation Expression" to macromolecular synthetic activities of a UV-irradiated culture of *Escherichia coli* strain WP2. Mutation fixation was determined by the addition of chloramphenicol (20 micrograms/ml) to the incubating culture at the indicated times and incubating for an additional 45 minutes before plating on minimal agar plates supplemented with 2.5 per cent Difco nutrient broth.[5] Mutation expression was determined by plating directly onto minimal agar plates after the indicated times of incubation in minimal medium supplemented with casein hydrolysate (2 mg/ml) and *dl*-tryptophan (0.2 mg/ml). Prototrophs are expressed as the per cent of the maximum mutation frequency response. Maximum mutation frequency response at the UV dose used here was approximately 80 prototrophs per 10^6 colony-forming organisms when the culture was plated on the broth-supplemented minimal agar plates, and approximately 60 prototrophs per 10^6 colony-forming organisms when plated on the unsupplemented minimal agar.

conclusion that the initial event in the mutation induction process involves the synthesis of RNA.

If, instead of plating on the supplemented medium, this same culture is plated onto minimal agar medium, few mutations appear during the first 40 minutes of incubation (*mutation expression*, Fig. 1). At this point the "mutation fixation" process is essentially complete. With additional incubation prior to plating, an increasing number of prototrophs appear on the minimal plates. After 90 minutes of incubation, the maximum response is obtained. The increasing number of mutations observed on minimal agar plates is closely correlated with the initial postirradiation synthesis of DNA in the culture (see Fig. 1).

An amino acid requiring auxotroph, when plated on minimal agar medium, is unable to synthesize protein. Therefore, these results suggest that protein synthesis subsequent to the initial postirradiation synthesis of DNA is essential to mutation expression on minimal agar medium. This hypothesis was tested by incubating the irradiated cells following initiation of DNA synthesis for different time intervals in the presence of chloramphenicol prior to plating. Chloramphenicol should block protein synthesis and thus prevent mutation expression as measured by plating onto minimal agar plates. This "late" chloramphenicol challenge should not promote a *decline* in mutation frequency comparable to that obtained when it is used prior to RNA synthesis in the culture.

TABLE 1

EFFECT OF "LATE" CHLORAMPHENICOL ADDITION ON MUTATION INDUCTION AND EXPRESSION IN *E. coli* STRAIN WP2 FOLLOWING UV EXPOSURE OF 960 ERGS/MM²

Treatment	Yield — Postirradiation Incubation Time, minutes*				
	60	70	80	90	100
Control					
Amt. of DNA relative to unincubated control	1.4	1.7	2.1	2.5	3.0
Mutation frequency† when plated on minimal agar	22	30	50	61	60
Mutation frequency† when plated on minimal agar supplemented with 2.5% broth	84	82	81	80	81
Chloramphenicol‡					
Amt. of DNA relative to unincubated control	1.4	1.7	2.0	2.5	2.9
Mutation frequency† when plated on minimal agar	22	26	24	25	25
Mutation frequency† when plated on minimal agar supplemented with 2.5% broth	84	85	84	82	80

* Postirradiation incubation is in liquid minimal medium supplemented with casein hydrolysate (2 mg/ml) and with *dl*-tryptophan (0.2 mg/ml).
† Prototrophs per 10^8 surviving organisms.
‡ Chloramphenicol (20 micrograms/ml) added after 60 minutes of incubation.

The results of an experiment of this type are given in Table 1. They show that DNA synthesis continues in the presence of chloramphenicol which is added to the culture after 60 minutes of incubation in amino acid supplemented medium. However, further mutation expression is prevented by the chloramphenicol when the cells are plated onto minimal agar medium at various intervals after chloramphenicol addition. The level of mutation expression, as demonstrated with this medium, remains constant after chloramphenicol addition for at least 40 minutes incubation in its presence. Since DNA synthesis is not interrupted by chloramphenicol, while mutation expression is prevented by it, the latter process must require post-DNA protein synthesis. This interval of incubation in chloram-

phenicol shown in Table 1 has no effect on mutation frequency response as deterphmined by plating the same cultures on nutrient broth containing medium. Therefore, it is probable that this "late" (post-RNA synthesis) chloramphenicol treatment has not interfered with the processes leading to mutation induction.

Discussion.—These experiments suggest that, while the processes upon which mutation induction depends occur prior to DNA synthesis, the expression of phenotypic change which is the product of the induced mutation follows the initial postirradiation synthesis of DNA. These results may explain recent observations which relate mutation frequency to DNA synthesis.[6] It thus appears reasonable that the final step in the process of mutation induction involves the synthesis of DNA.

The evidence suggests that reverted cells acquire the capacity for phenotypic expression of the "wild-type" state before they are plated on minimal agar which will allow no further protein synthesis in cells which have not acquired this capacity. This would account for the increasing frequency of prototrophs on minimal agar medium with postirradiation incubation in a complete medium before plating. It seems quite plausible that in these experiments we are measuring the initial action of a reverted gene. Therefore, the increase in mutation frequency observed on minimal agar after increasing periods of incubation in complete medium is probably related to the appearance of an enzyme involved in tryptophan synthesis and controlled by the mutated gene. If further work proves this to be the case, the technique should prove quite valuable for the study of the genetic control of enzyme formation since the processes involved in gene synthesis and mutation can be separated from those processes which are the product of gene action.

Summary.—(1) UV-induced reversion of an auxotrophic strain of *Escherichia coli* requiring tryptophan involves the postirradiation synthesis of DNA as the final step in the mutation induction process. (2) Mutation *expression* involves the synthesis of protein subsequent to postirradiation DNA synthesis. (3) With an amino acid deficient strain, the fraction of induced reversions capable of phenotypic expression at a given time following induction can be determined by plating identical aliquots of the cells on minimal agar and on minimal agar supplemented with low levels of the required amino acid. (4) It is suggested that this technique may serve as a means for differentiating those processes involved in gene synthesis and mutation from those involved in gene action.

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*THE RELATIONSHIP OF GENE CONVERSION TO CROSSING OVER IN NEUROSPORA**

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The production of infrequent wild-type progeny in crosses between strains carrying apparently allelic mutants of similar phenotype has been termed gene conversion. Mitchell[1] demonstrated in a cross between two pyridoxine mutants that conversion differed from crossing over in two ways: (1) the convertants did not have the expected array of closely linked markers; (2) the event was non-reciprocal; the ascus in which a *pdx*+ spore pair occurred did not contain a pyridoxine double mutant spore pair.

In spite of these differences, gene conversion and crossing over were correlated. The frequency of crossing over between the markers embracing the pyridoxine locus was only about 5 per cent in the general population, while 14 of the 32 *pdx*+ convertants had such crossing over. Mitchell suggested that gene conversion and crossing over were separate events and that they were correlated because both were favored by the same local conditions, as perhaps, especially close pairing of the homologous chromosomes.

A hypothesis whereby a single event results in both gene conversion and crossing over was formulated by Freese,[2] and this scheme was designed to account for the apparent differences between the two types of recombination reported by Mitchell. Freese states that gene conversion is observed for segregating genes *at the site* of the event which may result in crossing over for the surrounding markers. It is proposed that the recombination event is not a single exchange, but a switching of the replicating strands back and forth between the two templates, two or three or more times in a short interval. An odd number of switches results in crossing over with regard to the surrounding markers, while an even number of switches does not.

If conversion resulted from the same event as crossing over, it might be expected to have an effect associated with crossing over: interference with crossing over in neighboring regions. An experiment has been performed to study the relationship of gene conversion to crossing over in a separate but nearby chromosome region. The single event hypothesis would predict interference in all the convertants, even those with no recombination for the markers embracing the conversion locus. The separate events hypothesis would predict no interference in this group.

Materials and Methods.—Strains *lys*+ *cys-t ylo*+ *ad cot a* and *lys cys-c ylo ad*+ *cot A* were crossed on two sets of plates for which the procedures were identical except that they were allowed to mature at 25° in one case and at 18° in the other. The experiment was designed in this way in order to observe how the frequencies of gene conversion and of crossing over varied at the two temperatures.

Lys (lysine-requiring mutant DS6-85), *cys-t* and *cys-c* (cysteine mutants 80702 and 48401), *ylo* (yellow conidia, Y30539y), and *ad* (adenine mutant 3254) are all in the left arm of linkage group VI, and the order of the loci is *lys...cys...ylo...ad...*centromere.[3] *Cys-t* and *cys-c* are functionally allelic in that they do not complement each other in heterocaryons; however, crosses between them yield 2-3 *cys*+ asco-

spores per thousand by gene conversion.[4] The two cysteine mutants can be distinguished by nutritional responses at 25°C; *cys-t* will grow on either cysteine or thiosulfate, while *cys-c* will grown only on cysteine. The temperature colonial gene *cot*, present in both parents, results in the formation of small, dense colonies on agar plates at temperatures above 31°C, while growth at lower temperatures appears identical to wild-type.[5]

Random ascospores were suspended in 0.1 per cent agar and filtered through surgical gauze to remove clumps and debris. The suspension was then added to liquid 1 per cent agar at 60°C and held at this temperature for 25 minutes to accomplish activation of the spores. Four milliliter aliquots of the suspension (containing 1,000 to 5,000 ascospores) were poured over plates of minimal medium supplemented with lysine and adenine. Only the *cys+* convertants were capable of growth on this medium. The plates were incubated at 34° for six days, at which time all colonies were transferred to slants of complete medium and allowed to mature at 25°. Classification for *ylo* was noted, and growth tests were performed to score for *lys* and *ad*.

A *ten-fold* dilution of the ascospore suspension was plated over minimal medium supplemented with lysine, cysteine, and adenine. These plates were incubated six days at 34° and colonies were counted to determine the number of viable spores in the original suspension. Colonies from these plates were picked up and fully classified to determine crossing over frequencies in the general population.

TABLE 1

FREQUENCIES OF CYS+ CONVERTANTS

....lys+....cys-t....ylo+....ad.....
....lys.....cys-c....ylo.....ad+....

Genotype	25°	18°	Per 10^5 germinated spores 25°	18°
+ + + ad	112	52	42.6	30.4
lys + ylo +	201	147	77.0	85.8
+ + ylo +	155	98	59.4	57.2
lys + + ad	116	74	44.5	43.2
+ + + +	14	17	5.4	9.9
lys + ylo ad	34	24	13.0	14.0
+ + ylo ad	6	4	2.3	2.3
lys + + +	5	12	1.9	7.0
Total	643	428	246.0	250.0
Total germinated spores	260,800	171,200		

Results.—The frequencies of *cys+* convertants with the different combinations of linked markers are shown in Table 1. A comparison of the results of the cross which matured at 25° with those from the 18° cross reveals that the total conversion frequencies are very similar, and there are no striking differences in the frequencies of the various marker combinations.

The genotypes of randomly selected progeny are listed in Table 2. There is a sharp disparity between the numbers of complementary types in several classes; this results from the relative inviability of ascospores carrying the *cys-c* gene (particularly at 18°). There are no marked differences in crossing over frequency at the two temperatures, in contrast to other studies[3,6] which showed more crossing over at 18° than at 25°. One noteworthy aspect of the present data, however, is

TABLE 2

CROSSING OVER FREQUENCIES AMONG RANDOMLY SELECTED GERMINATED ASCOSPORES

....lys+....cys-t....ylo+....ad.....
I II III
....lys.....cys-c....ylo.....ad+....

Genotype	Region of Crossover	25°	18°
lys+ cys-t ylo+ ad	None	405	303
lys cys-c ylo ad+	None	79	20
lys+ cys-c ylo ad+	I	13	0
lys cys-t ylo+ ad	I	17	22
lys+ cys-t ylo ad+	II	103	62
lys cys-c ylo+ ad	II	18	3
lys+ cys-t ylo+ ad+	III	64	42
lys cys-c ylo ad	III	14	1
lys+ cys-c ylo+ ad	I, II	2	0
lys cys-t ylo ad+	I, II	7	1
lys cys-t ylo+ ad+	I, III	0	3
lys+ cys-t ylo ad	II, III	1	6
lys+ cys-c ylo+ ad+	I, II, III	1	0
Total		724	463

25° map: lys..5.5..cys..18.3..ylo..11.1..ad
18° map: lys..5.6..cys..15.5..ylo..11.2..ad

that the two crosses differ in patterns of multiple crossing over. Double exchanges involing the *lys-cys* and *cys-ylo* regions are more frequent in the 25° cross, while doubles in which one crossover takes place between *ylo* and *ad* occur more often at 18°.

The results are summarized with respect to interference with crossing over in the *ylo-ad* region in Table 3. The critical question is whether gene conversion results in such interference even in the absence of crossing over between *lys* and *ylo*. The answer is that it does not. In the 25° cross, the frequency of *ylo-ad* recombination among spores with parental combinations of *lys* and *ylo* is much the same (13.3% to 13.8%) whether or not gene conversion to *cys*+ has taken place. Among the corresponding types from the 18° cross the frequency of *ylo-ad* recombination among convertants is somewhat *higher* (less interference) than among non-convertants.

The results indicate that conversion differs from crossing over in that it does not interfere with crossing over in a nearby region. Is it possible that this is an erroneous conclusion resulting from some ambiguity of the experiment? Two aspects of the experimental setup may be suspect: the relative inviability of spores carrying the *cys-c* gene and the considerable crossing over distance between the conversion locus and the test region (*ylo-ad*).

Is the apparent interference among random spores a spurious result of the inviability of *cys-c*? No. In Table 4 the 25° data on interference are broken down into two groups according to which *cys* gene is present, and there is no apparent disparity between the two.

Does interference in the *ylo-ad* region take place only with crossing over to the right of the *cys* locus, which is itself too far away for such interference? This is plausible, as the map distance from *cys* to *ylo* is quite large (18.3% recombination at 25°). Were this the case, the absence of interference among *cys*+ convertants would not conflict with the single event hypothesis. In such a situation, however, this hypothesis would predict no interference in any of the convertants, whether the

TABLE 3

EFFECT OF GENE CONVERSION AT THE CYSTEINE LOCUS ON CROSSING OVER BETWEEN YLO AND AD

	ylo-ad recombination frequency	
	25°	18°
Cys+ convertants		
Parental combination of lys and ylo	48/361 (13.3%)	41/240 (17.1%)
non-parental combination of lys and ylo	11/282 (3.9%)	16/188 (8.5%)
Random spores		
parental combination of lys and ylo	79/572 (13.8%)	43/367 (11.7%)
non-parental combination of lys and ylo	1/152 (0.7%)	9/96 (9.4%)

TABLE 4

EFFECT OF THE CYSTEINE GENOTYPE ON INTERFERENCE (25° cross)

	ylo-ad recombination frequency		
	cys-c	*cys-t*	*total*
Parental combination of lys and ylo	15/96	64/476	79/572
Non-parental combination of lys and ylo	0/31	1/121	1/152

switching at the *cys* locus resulted in recombination for the surrounding markers or not. Clearly this prediction is not fulfilled (see Table 3).

Evidence that the *cys* locus is close enough to the *ylo-ad* interval for crossing over interference would be provided by a demonstration that such interference is manifest with crossing over in the *lys-ylo* region, which is even farther away. This appears to be the case, although the data are scant. In the 25° data of Table 2, the predicted number of coincident crossovers in regions I and III would be 4.4 in the absence of interference, while only one such event was observed.

The results of the 18° cross are less clear-cut, because there is only weak interference in the *ylo-ad* region even among *lys-ylo* recombinants. To the extent that they can be evaluated, these data tend to confirm the conclusion drawn from the 25° results.

Discussion.—Two general types of hypothesis have been advanced regarding the relationship of gene conversion to crossing over. (1) the two are separate events; (2) they are both results of a single recombination event. Several studies have resulted in findings which are difficult to explain by the single event hypothesis. Roman[7] observed mitotic conversion to wild-type in an adenine-requiring diploid yeast; the event was not accompanied by crossing over. Heat shock in Neurospora[8] and ultraviolet treatment in yeast[9] caused changes in conversion frequency without parallel changes in crossing over. Changing the genetic background resulted in a threefold increase in crossing over around the *cys* region in Neurospora without significant changes in the frequency or pattern of gene conversion at this locus.[4]

The present study has shown that gene conversion differs from crossing over in that it does not interfere with crossing over in a neighboring region. The single event hypothesis would not have predicted such a result, although the hypothesis could be readily modified in such a way as to account for this finding. It would seem, however, that the simpler assumption at the present time is that gene conversion and crossing over result from different events.

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*CONFORMALLY INVARIANT METRICS ON RIEMANN SURFACES**

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The object of this note is to assert the existence of two conformally invariant metrics, give the definitions, and describe the principal results with some indications of the proofs. By a metric d we shall mean a metric function in the topological sense so that d is a non-negative function on the product $X \times X$. Here the underlying set X is a Riemann surface and so it is a topological space with a locally Euclidean topology $\mathfrak{I}$. If we speak about a metric d for X we shall always tacitly assume that d is compatible with $\mathfrak{I}$, that is, the topology generated on X by d is $\mathfrak{I}$.

We shall define two pseudo-metrics d^i $(i = 1,2)$ on any Riemann surface X and save for a few exceptional types these functions d^i will turn out to be proper metrics compatible with the topology $\mathfrak{I}$. The definitions will be intrinsic and hence these metrics d^i will be invariant under conformal mappings: If the points x_1, x_2 are mapped into y_1, y_2 under a conformal map of X onto Y then $d^i_X(x_1, x_2) = d^i_Y(y_1, y_2)$ where d^i_X and d^i_Y denote the metrics given on X and Y, respectively.

The pseudo-metrics d^i will be defined by extending the notion of extremal length due to Ahlfors and Beurling (see ref. 1, pp. 114–115). The *extremal length* λ of a family Γ of curves γ on a Riemann surface X is defined as follows: Let ω be a pure first order differential on X, that is let $*\omega + i\omega = 0$ where $*\omega$ is the star conjugate of ω. Since ω is pure its modulus $|\omega|$ can be integrated along the curves γ of Γ. We let

$$L(\omega) = \underset{\gamma \epsilon \Gamma}{glb} \int_\gamma |\omega|$$

and

$$A(\omega) = \frac{i}{2} \int_X \omega \bar{\omega}.$$

The extremal length of the family Γ is defined as

$$\lambda = \lambda\{\Gamma\} = lub \frac{L(\omega)^2}{A(\omega)}$$

where the least upper bound is taken with respect to all infinitely differentiable ω's satisfying $A(\omega) < +\infty$. (We could also consider continuous or arbitrary integrable pure first order differentials.) In order to define the distances $d^i(x, y)$ $(i = 1, 2)$ of the points $x, y \epsilon X$ we consider simply connected regions S_{xy} of X containing x, y and such that the boundary of S_{xy} is a Jordan curve or a Jordan arc. We let Γ^1_{xy} be the family of all possible boundary curves and let Γ^2_{xy} be the subfamily of Jordan curves. Then *we define*

$$d^i(x, y) = \sqrt{\lambda^i_{xy}} \qquad (i = 1, 2)$$

where λ^i_{xy} *is the extremal length of the family* Γ^i_{xy}.

The fact that $d^i(x, y)$ is finite and defines a pseudo-metric on X is proved in three steps: First one proves the following restricted form of the triangle inequality: If $x_1, x_2, x_3 \epsilon X$ and if both $d^i(x_1, x_2)$ and $d^i(x_2, x_3)$ are finite then $d^i(x_1, x_3)$ is finite and

$$d^i(x_1, x_3) \leq d^i(x_1, x_2) + d^i(x_2, x_3).$$

This is followed by proving that λ^i_{xy} is finite provided y belongs to a sufficiently small neighborhood of $x \epsilon X$. Here one uses the monotonicity properties of the extremal length and the well-known formula which gives the extremal length of the family of simple closed curves separating the two boundary components of an annulus. The same reasoning also shows that the topology $\mathfrak{I}^i$ induced on X by the pseudo-metric d^i is at most as strong as τ. Finally, using a simple compactness argument one proves that $d^i(x, y)$ is finite for every $x, y \epsilon X$.

The *exceptional cases* are all trivial in the sense that if d^i is not compatible with τ then $d^i(x, y) = 0$ for every pair of points $x, y \epsilon X$. If X is conformally equivalent to the Riemann sphere or the finite plane then d^1 and d^2 are both trivial. Moreover if X is equivalent to the unit disk then d^1 is trivial while d^2 is compatible with the topology of X. In any other case both d^1 *and* d^2 *are compatible with the topology* $\mathfrak{I}$. In particular if the Riemann surface X is multiply connected, then d^1 and d^2 generate the topology on X. In general $\mathfrak{I}^1 \leq \mathfrak{I}^2 \leq \mathfrak{I}$ and so it is sufficient to prove that $\mathfrak{I}^1 > \mathfrak{I}$. Given $p \epsilon X$ and a neighborhood N_p of p one wishes to determine a $\delta \geq 0$ such that $d^1(p, q) < \delta$ only if $q \epsilon N_p$. This can be done by choosing an open set O_p containing a simple closed curve which is not homotopic to zero: If O_p is sufficiently small then it is schlichtartig and so it admits a global coordinate system. Using this coordinate system one determines a suitable pure form ω and a $\delta > 0$ satisfying the requirements. If X itself is schlichtartig the reasoning can be considerably simplified.

As far as uniform structures are concerned the metrics d^1 and d^2 behave differently. Let $\mathfrak{U}^i$ denote the *conformally invariant uniform structure* generated by d^i and let $\mathfrak{U}$ be the unique uniform structure compatible with the topology of the Riemann sphere S^2. The uniform structure induced on a region of S^2 will be denoted also by $\mathfrak{U}$. By a *Jordan domain* we understand a region of S^2 whose boundary components are finitely many simple closed curves.

THEOREM 1. *If X is a multiply connected Jordan domain then $\mathfrak{U}^1 = \mathfrak{U}$.*

In particular if X is a bounded Jordan domain in the finite plane then $\mathfrak{U}^1$ is the usual structure generated by the Euclidean metric $|p - q|$.

COROLLARY. *If X and Y are bounded Jordan domains in the finite plane and if f maps X conformally onto Y then f is uniformly continuous with respect to the usual structure of the plane.*

The uniform structures $\mathfrak{U}$ and $\mathfrak{U}^1$ of the regions of S^2 are in general distinct: $\mathfrak{U}$ and $\mathfrak{U}^1$ can be incomparable and the possibilities $\mathfrak{U} < \mathfrak{U}^1$ and $\mathfrak{U} > \mathfrak{U}^1$ are also realized. The proof of the above theorem is elementary and only the simplest properties of Jordan domains are used. First one proves that if the bounded plane region X is multiply connected and if its boundary consists of finitely many isolated points and simple closed curves then $\mathfrak{U} \leq \mathfrak{U}^1$. Then one shows that for any Jordan domain $\mathfrak{U}^1 \leq \mathfrak{U}$.

THEOREM 2. *If X and Y are Jordan domains and if $f: X \to Y$ is a conformal map then f can be extended in a unique way to a one-to-one map $\bar{f}$ between the closures of X and Y in S^2 such that both $\bar{f}$ and its inverse are continuous maps.*

A special case of this theorem states that if X and Y are bounded domains in the finite plane then $\bar{f}$ and its inverse are continuous in the usual sense. Therefore the result is an extension of Carathéodory's theorem to Jordan domains of higher connectivity. For simply connected Jordan domains two proofs were known earlier: Carathédory's original proof is based on Fatou's theorem concerning the existence of radial limits of harmonic functions (see ref. 3). A later proof avoids Fatou's theorem but instead a result of Lindelöf is used (see ref. 4, pp. 80–86). In order to obtain Theorem 2 one proves a lemma on the continuous extension of uniformly continuous maps: Let X and Y be uniformizable Hausdorff spaces with uniform structures $\mathfrak{U}_x$ and $\mathfrak{U}_y$, respectively. It is known that if $f: X \to Y$ is uniformly continuous then f can be extended in a unique manner to a uniformly continuous map $\bar{f}: \bar{X} \to \bar{Y}$ between the completions $\bar{X}$ and $\bar{Y}$ of X and Y, respectively. We need the following

LEMMA. *If $f: X \to Y$ is invertible and if both f and f^{-1} are uniformly continuous then $\bar{f}$ is a one-to-one map of $\bar{X}$ onto $\bar{Y}$ and both $\bar{f}$ and its inverse are uniformly continuous maps.*

Theorem 2 is a simple consequence of Theorem 1 and the Lemma.

It is possible to extend the definition of the uniform structures $\mathfrak{U}^i$ $(i = 1, 2)$ to simply connected Riemann surfaces such that the uniform topology generated by $\mathfrak{U}^i$ is always the original topology 3. For one defines two conformally invariant uniform structures $\mathfrak{V}^i$ $(i = 1, 2)$ which are compatible with the topology of the Riemann surface X and one proves that if $\mathfrak{U}^i$ is compatible with the topology of X then $\mathfrak{V}^i = \mathfrak{U}^i$. The uniform structure $\mathfrak{V}^i$ is defined as the least upper bound of a family of uniform structures $\mathfrak{V}^i(C, K)$ which can be described by specifying a base $\mathfrak{B}^i(C, K)$: We choose a compact set C, a compact neighborhood K of C, a real

number $\epsilon > 0$ and a vicinity U of the unique uniform structure compatible with the topology of K. Using these entities we define a vicinity $V^i = V^i(C, K, U, \epsilon)$ of $\mathfrak{B}^i(C, K)$ as follows:

$$V^i = [(x, y) : (x, y) \epsilon U \text{ or } d^i_{X-C}(x, y) < \epsilon].$$

One can easily show that if X is the unit disk then $\mathfrak{V}^1 = \mathfrak{U}$. It follows that if X is simply connected then $\mathfrak{V}^1$ is the Carathéodory structure, i.e., it is the restriction to X of the unique uniform structure of the compactification of X by the use of prime ends. Thus $\mathfrak{V}^1$ is a natural extension of the Carathéodory structure to arbitrary Riemann surfaces. Classes of equivalent Cauchy filters or nets can be considered as prime ends associated with X. If X is not equivalent to the Riemann sphere or the finite plane this definition of a prime end is in accordance with another definition introduced on heuristic grounds earlier by Ahlfors (see refs. 5 and 6).

Clearly $d^1(x, y) \leq d^2(x, y)$ for any Riemann surface and so $\mathfrak{U}^1 \leq \mathfrak{U}^2$. Hence by Theorem 1 we have $\mathfrak{U} \leq \mathfrak{U}^2$. One can prove the following stronger

THEOREM 3. *If the schlichtartig Riemann surface X is not simply connected then $\mathfrak{U}^2$ is complete and it is strictly finer than $\mathfrak{U}$.*

It follows that the uniform structure $\mathfrak{U}^2$ of a schlichtartig Riemann surface X is never precompact. The proof is based on a simple lower estimate of $d^2(x, y)$ in terms of the distance d_{xy} of the point pair $x, y \neq \infty$ from the boundary of X. For we have

$$d^2(x, y) > \frac{|x - y|}{2|x - y| + d_{xy}}$$

and if $y = \infty$ then

$$d^2(x, y) > \frac{1}{2 + \delta_{xy}|x|}$$

where δ_{xy} is the distance of the boundary of $[x^{-1} : x \epsilon X]$ from the pair $x^{-1}, 0$. These inequalities can be easily proved by choosing a suitable pure differential ω on X.

In the sequel let Δ denote the unit disk $[z : |z| < 1]$. Using the foregoing lower estimates one can prove that the sets

$$U_\epsilon = [(x, y) : |x - y| < d_{xy}] \qquad (0 < \epsilon < 1)$$

form a base for the uniform structure $\mathfrak{U}^2$ of Δ. It follows that if the complex analytic function f is bounded on the disk Δ then f is uniformly continuous with respect to the uniform structure $\mathfrak{U}^2$. Moreover using the familiar expression for the line element in the Poincaré model we see that the hyperbolic metric and the conformally invariant metric d^2 generate the same uniform structure on the disk Δ. We can prove that this conformally invariant uniform structure is the weakest uniform structure for which the family of functions $f_\theta : z \to \log(e^{i\theta} + z)$ is uniformly equi-continuous.

The uniform structure $\mathfrak{U}^1$ is not necessarily precompact. For instance if X is the punctured disk $[z : 0 < |z| < 1]$ then in the neighborhood of $z = 0$ the metrics d^1 and d^2 coincide and so by Theorem 3 the completion of X with respect to $\mathfrak{U}^1$ is $[z : 0 < |z| \leq 1]$.

The metric function d^2 can be used to introduce a *Riemannian metric* on the Riemann surface X. Let X be conformally equivalent to a bounded domain B in the finite plane and let $K(z, w)$ be the Bergman kernel function of B. It can be easily proved that

$$\frac{d^2(z, z + \Delta z)}{|\Delta z|} \to \sqrt{K(z, \bar{z})}$$

as $\Delta z \to 0$. In fact it is sufficient to notice that $K(z, \bar{z})$ is the solution of an extremal problem formulated by Bergman (see ref. 2, pp. 21–22). Therefore the Riemannian metric generated by the metric d^2 is the conformally *invariant metric of Bergman.*

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INVARIANT SETS OF STRUCTURALLY STABLE DIFFERENTIAL SYSTEMS

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Let M^n be a compact, differentiable (C^∞) Riemann manifold and let $\mathfrak{B}$ be the Banach space of all C' contravariant vector fields on M^n, using the C'-norm. A vector field, or differential system, $S \in \mathfrak{B}$ is called structurally stable in case: for each $\epsilon > 0$ there exists $\delta > 0$ such that, whenever $S' \in \mathfrak{B}$ and $\|S - S'\|_1 < \delta$, then there is an ϵ-homeomorphism of M^n onto itself carrying the sensed (not parametrized) solution curves of S onto those of S', and vice versa. The slight modifications, for the case when S is defined in an open subset $\mathcal{O} \subset M^n$, are considered in an article to appear soon (reference 3, also 1, 4). Thus we assume that S is structurally stable on a compact Riemann manifold M^n.

THEOREM 1. *There are a finite number of critical points of S on M^n and each of these is isolated, elementary, and topologically linear.*

That is, write S: $\dot{x}^i = f^i(x)$ in local coordinates near a critical point $P: x^i = 0$ and then the eigenvalues of $\dfrac{\partial f^i}{\partial x^j}(0) = a^i_j$ have non-zero real parts. Furthermore there is a homeomorphism of S near P onto $\dot{x}^i = a^i_j x^j$ near the origin in the number space R^n.

THEOREM 2. *The periodic solutions of* $\mathcal{S}$ *in* M^n *are isolated, elementary, and topologically linear.*

That is, each periodic solution S of $\mathcal{S}$ lies in a tubular neighborhood N which contains no other periodic solution of $\mathcal{S}$. Also the eigenvalues for the transversal germ of S have non-unit moduli. Furthermore there is a homeomorphism of $\mathcal{S}$ in N onto a neighborhood of a periodic solution S', of a C^∞ differential system $\mathcal{S}'$ in an anchor ring or Klein bottle, and S' has a linear transversal germ.

COROLLARY. *For a given bound* $T > 0$ *there are only a finite number of periodic solutions of* $\mathcal{S}$ *with period less than* T. *Thus there are at most a countable number of periodic solutions of* $\mathcal{S}$ *in* M^n.

It is not known if there exist structurally stable systems with infinitely many periodic solutions. However the geodesic flow on the tangent sphere bundle of a Riemann manifold of constant negative curvature is a possible candidate.

THEOREM 3. *Each limit set* $S+$ *of a solution of* $\mathcal{S}$ *in* M^n *is either a critical point, a periodic solution, or every neighborhood of* $S+$ *contains infinitely many periodic solutions of* $\mathcal{S}$ *with arbitrarily long (minimal) periods.*

COROLLARY 1. *If* $\mathcal{S}$ *has only a finite number of periodic solutions, then each limit set* $S+$, *and hence each minimal set* K *of* $\mathcal{S}$, *is either a critical point or a periodic solution. In this case there exists either a stable critical point or a stable periodic solution of* $\mathcal{S}$ *on* M^n.

COROLLARY 2. *If* $\mathcal{S}$ *has no critical points on* M^n, *then* $\mathcal{S}$ *has at least two periodic solutions on* M^n.

In case M^n is the 3-sphere our corollary 2 is relevant to a problem of H. Seifert.[5]

The general topological theory[2] of structurally stable systems is simplified by the next result.

THEOREM 4. *The non-wandering motions of* $\mathcal{S}$ *are exactly the central motions* M_τ. *Furthermore the set of critical points and periodic solutions of* $\mathcal{S}$ *is dense in* M_τ; *and, when there are only a finite number of periodic solutions, it is all of* M_τ.

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ON DISTRIBUTION PROBLEMS INVOLVING THE NUMBERS OF SOLUTIONS OF CERTAIN TRINOMIAL CONGRUENCES

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In several previous papers[1] certain tables were discussed involving the number of solutions of the congruence

$$1 + ax^c \equiv by^l \pmod{p}, \tag{1}$$

where $cl + 1 = p$, and $xy \not\equiv 0 \pmod{p}$, with a and b fixed initially. A primitive root of p, say g, was then introduced, and then the problem obviously reduces to the consideration of the values of r and s in the congruence

$$1 \equiv g^{j+ls} + g^{i+cr} \pmod{p}, \tag{2}$$

where i and j are fixed, $0 \leqq i \leqq c - 1$; $0 \leqq j \leqq l - 1$. The number of sets r,s, which satisfy (2), we designate by $[i,j]_{cl}$, but since c and l are fixed throughout in this article we abbreviate this to $[i,j]$. This problem (first formulated in paper I) was taken up on the SWAC,[2] the rapid digital computing machine of the National Bureau of Standards at the Institute for Numerical Analysis in Los Angeles, California, and the results tabulated for $l = 5$ for each $p \equiv 1 \pmod{5}$ for $p < 1024$; also for $l = 7$ with all p's < 1024; $p \equiv 1 \pmod{7}$. Results of these tables were analyzed in paper II. In paper III we indicated how a study of the tables and the analyses of them led to new conjectures and theorems concerning the trinomial congruences. In particular, we mentioned (paper III, p. 462–463, the paragraph following the statement of the theorem on page 463) a conjecture which we stated we had not been able to prove in full. In the present paper we continue this discussion.

The conjecture just mentioned is as follows: If l is fixed and p is any sufficiently large prime, and if

$$b_0 + b_1 + \ldots + b_{l-1} = l$$

is any partition of l into non-negative parts for a fixed $i \not\equiv 0 \pmod{c}$ in (2), then it is possible to select an i in the set $0, 1, \ldots, c - 1$ such that the numbers $[i,j]$, $j = 0, 1, 2, \ldots, l - 1$ form a permutation of $b_0, b_1, \ldots, b_{l-1}$. Attempts to prove this led to a related result which is stated and proved here (Theorem I).

Set, if $N(\mathfrak{p})$ is the norm of $\mathfrak{p}$, and $(N(\mathfrak{p}) - 1)/l = M$,

$$\left(\frac{\omega}{\mathfrak{p}}\right) \equiv \zeta^M \pmod{\mathfrak{p}},$$

where ω is an integer in $K(\zeta)$, $\zeta = e^{2i\pi/l}$ where l is an odd prime, $((l),\mathfrak{p}) = 1$, $\mathfrak{p}$ is a prime ideal in K, prime to (ω), such that $g^l \equiv \zeta \pmod{\mathfrak{p}}$, which divides (p) with p a rational prime, $p^n = 1 + cl$. Throughout this paper small Latin letters denote rational integers. By a theorem given by Takagi[3] there exists an infinity of prime ideals $\mathfrak{p}$ of the first degree in K (so that we may take $n = 1$ in $p^n = 1 + cl$) such that if

$$1 + t\zeta^i = \alpha_i;\ i = 0, 1, \ldots, l-1,$$

with

$$((1 + t\zeta^i), \mathfrak{p}) = 1, \tag{2a}$$

$$((1 + t\zeta^i), (l)) = 1, \tag{2b}$$

$$\left(\frac{\zeta}{\mathfrak{p}}\right) \not\equiv 1, \left(\frac{\alpha_0}{\mathfrak{p}}\right) = \zeta^{a_0 n} \left(\frac{\alpha_1}{\mathfrak{p}}\right) = \zeta^{a_1 n}, \ldots, \left(\frac{\alpha_{l-1}}{\mathfrak{p}}\right) = \zeta^{a_{l-1} n}, \tag{3}$$

where the a's are prescribed integers in the set $0, 1, \ldots, l-1$; and $n \not\equiv 0 \pmod{l}$, provided that in the relation

$$\zeta^d \alpha_0^{m_0} \alpha_1^{m_1} \ldots \alpha_{l-1}^{m_{l-1}} = \gamma^l, \tag{4}$$

with γ an integer in K, then $m_i \equiv 0 \pmod{l}$; $i = 0, 1, \ldots, l-1$, and $d \equiv 0 \pmod{l}$. From (3) we must have

$$t \not\equiv 0 \pmod{\mathfrak{p}}. \tag{5}$$

We know by the definitions above that $((1 + t^l), \mathfrak{p}(l)) = 1$. We shall now find under what conditions the relation (4) holds. We first note that if $i \not\equiv 0 \pmod{l}$, $(1 + t\zeta^i)$ is prime to $1 + t$ since, if they have a prime ideal factor $\mathfrak{p}$, then $1 + t\zeta^i \equiv 0 \pmod{\mathfrak{p}}$ and $1 + t \equiv 0 \pmod{\mathfrak{p}}$ give from (5),

$$1 - \zeta^i \equiv 0 \pmod{\mathfrak{p}}, \tag{6}$$

or $\mathfrak{p}$ divides (l) contradicting $(1 + t^l, l) = 1$.

Now consider the relation (4) since by (6) the α's are prime each to each, we have

$$(1 + t\zeta^j)^{m_j} = \mathfrak{a}_j^l;\ j = 0, 1, \ldots, l-1, \tag{7}$$

with $\mathfrak{a}$ an ideal[4] in K. Assume $m_j \not\equiv 0 \pmod{l}$ for a particular j in (7). Set, in lieu of ζ in (7), $\zeta^2, \zeta^3, \ldots, \zeta^{l-1}$, when $j \neq 0$. We then obtain $l - 1$ equations, and multiplying them all together we obtain

$$v^{m_j} = w^l,$$

where $v = (1 + t^l)/(1 + t)$. From this we obtain, since we assumed $m_j \not\equiv 0 \pmod{l}$, $v = w_1 l$; consequently, if we assume instead of this,

$$v \not\equiv w_1^l, \tag{8}$$

then all the m_j's, $j \neq 0$, are divisible by l. (We note that (8) is satisfied if $t = \pm 1$. In that case $1 + t\zeta$ becomes a unit or a divisor of l, the latter case contradicting $((1 + \zeta t), (l)) = 1$).

Using the fact that each of the m_j's, $j \neq 0$, is divisible by l, then (4) gives, with α, $\beta \epsilon K$,

$$(t + 1)^{m_0} \beta^l \zeta^d = \alpha^l, \tag{9}$$

and

$$((t + 1)^{m_0}) = \mathfrak{b}^l.$$

Whence, using the assumption that $(m_0, l) = 1$, we have

$$(t + 1) = \mathfrak{b}_1^l,$$

or $t + 1 = u^l$. Hence $m_0 \equiv 0 \pmod{l}$ provided

$$t + 1 \not\equiv u^l. \tag{10}$$

We shall now show that $d \equiv 0 \pmod{l}$ in (4). From what we have shown about the m's in it we have, if $\beta, \gamma \epsilon K(\zeta)$,

$$\zeta^d \beta^l = \gamma^l, \tag{10a}$$

with β, γ in $K(\zeta)$. Put $\zeta = 1 + \lambda$; $\lambda = \zeta - 1$. We have $\zeta^d \equiv 1 + d\lambda \pmod{\lambda^2}$, $\beta^l \equiv \gamma^l \equiv a \pmod{\lambda^l}$. This gives $d \equiv 0 \pmod{l}$, so all the exponents in (4) are divisible by l.

In place of the condition

$$\frac{1 + t^l}{1 + t} \not\equiv w_2^l, \tag{11}$$

we might mention that it could be replaced by a weaker condition which, however, is easier to apply when t is large. Assume that

$$t^{l-1} \not\equiv 1 \pmod{l^2}, \tag{12}$$

and also

$$t \not\equiv 0 \pmod{l}. \tag{13}$$

In view of the relation (7) we have, with $j \not\equiv 0$,

$$\left(\frac{\zeta}{1 + t\zeta^j}\right)^{m_j} = 1. \tag{14}$$

Whence

$$m_j \frac{N(1 + t\zeta^j) - 1}{l} \equiv 0 \pmod{l}, \tag{15}$$

so that

$$\frac{N(1 + t\zeta^j) - 1}{l} \equiv \frac{t^l + 1 - (t + 1)}{l(t + 1)} \equiv \frac{t(t^{l-1} - 1)}{l} \pmod{l}. \tag{16}$$

Using (12), (13), and (15) we find $m_j \equiv 0 \pmod{l}$ for $j = 1, 2, \ldots, l - 1$. Hence all the m's in (4) $\equiv 0 \pmod{l}$ under the assumptions (10) and (11) or (12) and (13), in addition to (2a), (2b), and (5) in each case.

The condition in (3),

$$\left(\frac{\zeta}{\mathfrak{p}}\right) \not\equiv 1,$$

gives

$$\frac{N(\mathfrak{p}) - 1}{l} \not\equiv 0 \pmod{l},$$

or

$$c \not\equiv 0 \pmod{l}.$$

Hence we may write

$$\left(\frac{\zeta}{\mathfrak{p}}\right) = \zeta^{kn},$$

$(kn,l) = 1$. Since $(c,l) = 1$, we may write $cf \equiv 1 \pmod{l}$,

$$\left(\frac{\alpha_i}{\mathfrak{p}}\right) = \zeta^{a_i fc}, \tag{17}$$

$i = 0, 1, \ldots (l-1)$, and $fc \equiv n \pmod{l}$. This gives

$$(1 + t\zeta^i)^c \equiv \zeta^{a_i fc} \pmod{\mathfrak{p}},$$

$$(\zeta^{-a_i f}(1 + t\zeta^i))^c \equiv 1 \pmod{\mathfrak{p}},$$

Whence, for $i = 0, 1, \ldots, l-1$,

$$1 + t\zeta^i \equiv \zeta^{a_i f}\rho_i^{\,l} \pmod{\mathfrak{p}}. \tag{18}$$

Now consider the integers which are incongruent, modulo l, in the set,

$$cfa_0,\ cfa_1,\ cfa_2,\ \ldots,\ cfa_{l-1}.$$

If $0 \leqq h_s < l$, let them be congruent to

$$h_1,\ h_2,\ \ldots,\ h_u.$$

Set $t \equiv -g^v \pmod{p}$ where g, as before, is a primitive root of p, and since p is of the first degree, then g is also a primitive root of $\mathfrak{p}$. Using (18) and

$$g^c \equiv \zeta \pmod{\mathfrak{p}},$$

we obtain

$$g^{v+cd} + g^{h_k+le_d} \equiv 1 \pmod{p}, \tag{19}$$

where $0 \leqq v < c$, d is in the range $0, 1, \ldots, l-1$, and e_d is in the range $0, 1, \ldots, c-1$. We shall now note that the number of possible sets (d,e_d) in (19) is $[v,h_k]$, for we observe that $[i,j]$ was defined as the number of sets (r,s) which satisfy our relation (2), and in (19) d is any number in the set $0, 1, \ldots, l-1$, since i in (18) was so defined. Also, there is an e_d corresponding to each d in (19) since $g^{v+cd} \equiv 1$ gives $v \equiv 0 \pmod{c}$, contradicting $t \not\equiv 1$ in (11).

Let

$$b_0 + b_1 + \ldots + b_{l-1} = l, \tag{20}$$

with the b's given integers in the set $0, 1, \ldots, l-1$.

By considering the relations found from (19), by letting d range over all the values $0, 1, \ldots, l-1$, and adding, we have

$$\sum_k [v,h_k] = l,$$

since there are l distinct values for d. Also, there are u distinct values for k. It may be that there is an integer j in the set $0, 1, \ldots, l-1$, which is not included in our set of h's, in which case we write $[v,j] = 0$. Consequently the total number of solutions of the set (19), by taking d over the set $0, 1, \ldots, l-1$, is

$$\sum_{j=0}^{l-1}[v,j],$$

and by a known result[5] this equals l. Also, since the a_i's in (18) were arbitrary, so we may set $[v,j] = b_j$ in (20), $j = 0, 1, \ldots, l-1$. Since the conditions (2a) and (5) hold for p sufficiently large unless $1 + t_l = 0$ and $t = 0$, we have

THEOREM 1. *Let l be an odd prime and t a fixed rational integer, and let (20) be any partition of l into non-negative parts less than l; then there exist infinitely many primes $p = cl + 1$, $c \not\equiv 0 \pmod{l}$ such that if g is any primitive root of p then if $g^v \equiv t \pmod{p}$,*

$$[v,0],\ [v,1],\ \ldots,\ [v,l-1] \tag{21}$$

is the set of b's in some order provided it satisfies none of the relations

$$t^l + 1 \not\equiv 0 \pmod{l},\ t \neq 0,\ t+1 \neq u^l, \text{ and } \frac{(t^l+1)}{t+1} \neq w^l. \tag{22}$$

the latter two having been introduced in (8) and (10). The symbols in (21) are defined just below our relation (2).

Consider again the conjecture made concerning the b's near the beginning of this paper. Theorem I does not prove said conjecture, but for certain p's the former is more general.

Theorem 1 may be generalized. The proof of this will be indicated only. To do this we may replace equation (3) by a more extended set of relations in lieu of the single set of relations involving $\alpha_0, \alpha_1, \ldots, \alpha_{l-1}$ with ζ, and we can use the sets

$$\alpha_{0k},\ \alpha_{1k},\ \ldots,\ \alpha_{l-1,k},$$

$k = 0, 1, \ldots, w$, where $\alpha_{i0} = \alpha_i$; $i = 0, 1, \ldots, l-1$; and where

$$T(i,k) = 1 + t_k\zeta_i, \tag{22a}$$

with $T(i,0) = \alpha_i$; $i = 0, 1, \ldots, l-1$; and where the lw quantities in (22a) are prime each to each. Also, for the norm of $\mathfrak{p}$ sufficiently large, each $t_k \not\equiv 0 \pmod{\mathfrak{p}}$, and $t_k + \zeta_s t_h \not\equiv 0 \pmod{\mathfrak{p}}$ for s arbitrary unless $t_k = 0$ or $t_k + \zeta^s t_h = 0$.

In (3), retain the relation

$$\left(\frac{\zeta}{\mathfrak{p}}\right) \neq 1,$$

and replace the others by the elements of the set of sets,

$$\left(\frac{T(0,k)}{\mathfrak{p}}\right) = \zeta^{A(0,k)}, \left(\frac{T(1,k)}{\mathfrak{p}}\right) = \zeta^{A(1,k)}, \ldots, \left(\frac{T(l-1,k)}{\mathfrak{p}}\right) = \zeta^{A(l-1,k)}, \tag{23}$$

where $k = 0, 1, \ldots, w$, and

$$A(i,k) = a_{ik}n_k$$

with $i = 0, 1, \ldots, l-1$; the a's are arbitrary and $(n_k, l) = 1$.

Corresponding to the relation (4), we have

$$\zeta^d \prod_{k=0}^{w} T(0,k)^{m_{0k}} T(1,k)^{m_{1k}} \ldots T(l-1,k)^{m_{l-1,k}} = \gamma_k{}^l. \tag{24}$$

Now to extend our argument already given in connection with equation (4) when we proved that each m_i was $\equiv 0 \pmod{l}$, we have to show that each m_{ik} is divisible by l, and $d \equiv 0 \pmod{l}$. This depends on the assumption we made that the quantities $T(j,k)$ are prime each to each for $j = 0, 1, \ldots, l-1$, and $k = 0, 1, \ldots, w$. In this connection we may state it is possible to select any number of t_k's such that the resulting $T(j,k)$'s are prime each to each.

In view of what we have just observed, we may then derive

$$(1 + t_k \zeta^j)^{m_{ik}} = \mathfrak{a}_{jk}{}^l \tag{25}$$

for each j and k and find, in the same way that equation (8) was obtained, that each exponent aside from d in (24) is divisible by l provided, if $k = 0, 1, \ldots, w$,

$$(1 + t_k{}^l)/(1 + t_k) \neq w_k{}^l.$$

In the same way that (10a) was obtained, we find that d is divisible by l in (24). We may then extend immediately the work in deriving (18) to the case where t_k replaces t throughout.

In view of the above results and in place of the statement in Theorem 1 concerning one partition of l, we may then replace this by considering r partitions of l in lieu of the bracket symbols in (21). We can replace them by w sets,

$$[v_k, 0],\ [v_k, 1],\ \ldots,\ [v_k, l-1],$$

for $k = 1, 2, \ldots, w$ where $g^{v_k} \equiv t_k \pmod{\mathfrak{p}}$, and the relations (22) are replaced by the relations

$$\left.\begin{aligned} t_h \neq 0,\ t_h{}^l \neq t_k{}^l \text{ for } h \neq k, \\ t_k{}^l + 1 \not\equiv 0 \pmod{l},\ t_k + 1 \neq u_k{}^l, \text{ and } \frac{t_k{}^l + 1}{t_k + 1} \neq w_k{}^l, \end{aligned}\right\} \tag{26}$$

where if $i = 0, 1, \ldots l-1$ and $k = 0, 1, \ldots, w$, then the quantities $T(i,k)$ are prime each to each.

If we consider the possibility of obtaining complementary theorems to Theorem 1 which provide for the exceptional cases noted in (22), we will find that to do this it seems necessary to engage in quite complicated discussions concerning the properties of units in $K(\zeta)$. However, there is a case where one of the conditions in (22), namely the third, is not satisfied, yet Theorem 1 is true for a particular partition of l, namely where all but one b are zero in (20). For in a former paper[6] it was shown that if $\lambda = (\zeta - 1)$ and $\mathfrak{p}$ is a principal ideal prime of the form $(1 + 2\lambda^l\theta)$, $\theta \epsilon K(\zeta)$, and for $t = 1$ the fourth condition in (22) is not satisfied, then

$$\left(\frac{1 + \zeta^a}{\mathfrak{p}}\right) = 1$$

for $a = 0, 1, \ldots, l-1$. It was pointed out that for $l = 3$, the primes $p = 109$ and 127 apply here, and for $l = 5$. a suitable value for p is 61051.

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[3] Takagi, T., "Ueber eine Theorie des relativ-Abelschen Zahlkörpers," *J. Coll. Sci., Imp. Univ. Tokyo*, **61**, 16 (1920).

[4] If the left-hand member of (7) is a unit, then (7) still holds, since then a is the unity ideal.

[5] Due to Gauss for $l = 3$. It is included as a special case of a relation given by Mitchell, H. H., *Proc. Amer. Math. Soc.*, **17**, 167 (1916), involving the number of solutions of trinomial equations in finite fields.

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*SPECTROPHOTOMETRIC STUDIES OF THE EFFECTS OF NITROGEN ON SOYBEAN NODULE EXTRACTS**

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Shug, Hamilton, and Wilson[1] and Hamilton, Shug, and Wilson,[2] demonstrated changes in the absorption spectra of sonic extracts of *Clostridium pasteurianum*, *Azotobacter vinelandii*, and soybean root nodules, when the extracts were placed under atmospheres of He, H_2, and N_2. With the nodule extracts these changes corresponded to the reduction and oxidation of the hemoglobin component. This paper presents data which support the specificity of the hemoglobin oxidation in the presence of N_2 and demonstrates the ability of the bacterial component of the nodules to reverse this effect.

Materials and Methods.—The methods used were based on those of Hamilton, Shug, and Wilson[2] and all precautions detailed by these authors were observed. The following details differ from those previously described. Nodules aged 4–5 weeks from glasshouse grown Lincoln variety soybeans inoculated with Strain CC711 of *Rhizobium japonicum* were used. Attempts to use nodules frozen and stored under H_2 were not successful, and extracts were prepared within one hour of nodule harvest, by crushing 2–3 gm. under a stream of H_2 in $M/15$ phosphate buffer pH 7.0 saturated with H_2. All manipulations were done at temperatures between 0° and 4°C. The crushed material was placed in the sonic field of a Raytheon 10-kc magnetostrictive oscillator for 3–5 minutes in a H_2 atmosphere. Longer sonic treatment reduced the activity of the extracts. After centrifugation at 7,000 × g for 15 minutes in tubes closed under a stream of H_2, the extracts were immediately transferred to 1 ml or 3 ml Pyrex cuvettes of 1 cm light path, fitted with Thunberg caps, and quickly evacuated. The gas space was then flushed ten times with

scrubbed He to remove the last traces of air. Storage of these preparations at $-20°$ under H_2 or He resulted in rapid loss of activity with respect to N_2, although the H_2 effect remained after 24 hours storage. Consequently all the experiments reported here were completed within 2 hours of the removal of nodules from the plants.

Commercial high purity helium, hydrogen, and nitrogen were used after being freed from contaminating O_2 by scrubbing with a solution of chromous chloride: (25% w/v $CrCl_3$ in 2*N* HCl reduced by adding Zn dust in an atmosphere of N_2 in a flask; when fully reduced the chromous chloride solution was transferred under vacuum to the scrubbing towers). In addition to scrubbing, N_2 was stored over chromous chloride solution in an aspirator bottle for at least 24 hours before use. Vessels were evacuated and filled from a vacuum line equipped with a mercury manometer and permanently connected with the gas supplies through scrubbing towers. Between manipulations all gas lines were flushed ten times with scrubbed He.

Gas mixtures containing small amounts of O_2 were obtained with a capillary gas buret calibrated in hundredths of a milliliter attached to a 2 liter bulb and connected to the vacuum line. Air in the buret was added to the scrubbed gas in the bulb by raising mercury in the capillary.

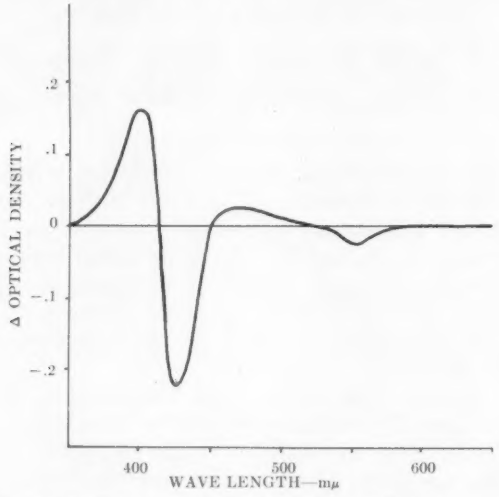

Fig. 1.—Difference spectrum of soybean nodule sonic extract. Reference cuvette He, sample-scrubbed N_2. The baseline was obtained with both cuvettes containing scrubbed He.

Spectra of clear extracts were obtained with a Beckman model DK2 ratio recording spectrophotometer and with a Beckman Model DU for extracts to which suspensions of bacteroids were added. In all cases the absolute and difference spectra between cuvettes were obtained for extracts under He. Preparations which contained more than a trace of hemiglobin were discarded, as also were preparations whose He—He difference spectra departed markedly from a straight

line. Slight deviations were compensated by replotting the curves obtained after changing the gas atmosphere.

Here, as elsewhere (Bergersen[3]), the term bacteroid is used to denote the intracellular forms of nodule bacteria which fill the central tissue of nitrogen-fixing nodules. Suspensions of bacteroids were obtained as previously described[3] from crushed nodules and washed in five changes of *M*/15 phosphate buffer pH 7.0.

Results.—Active nitrogen-fixing nodules yielded sonic extracts in which the hemoglobin was oxidized in the presence of scrubbed N_2 (Fig. 1). Occasional inactive preparations were attributed to the use of nodules older than 6 weeks, failure to maintain adequate exclusion of air during preparation and undue delays between steps. The difference spectra obtained between N_2 and He reached maximum values within 5 minutes from the final N_2 flushing and agitation(Table 1). When preparations oxidized in this way were flushed with H_2, the difference spectra indicated reduction of the pigment to give a more reduced form than in the preparation under He. Extracts allowed to stand for 4 hours under gas atmosphere containing 25 p.p.m. O_2 showed considerable oxidation of the pigment to hemiglobin due to the action of polyphenol oxidase in the preparation. Flushing these oxidized extracts with hydrogen had no effect.

TABLE 1

THE DEVELOPMENT OF THE N_2—He DIFFERENCE SPECTRUM OF SOYBEAN NODULE EXTRACTS

Wavelength, mμ	Δ OD at 1 min.	3 min.	5 min.	10 min.
400	+0.042	+0.110	+0.112	+0.113
425	−0.050	−0.110	−0.113	−0.113
555	0.000	−0.010	−0.018	−0.018

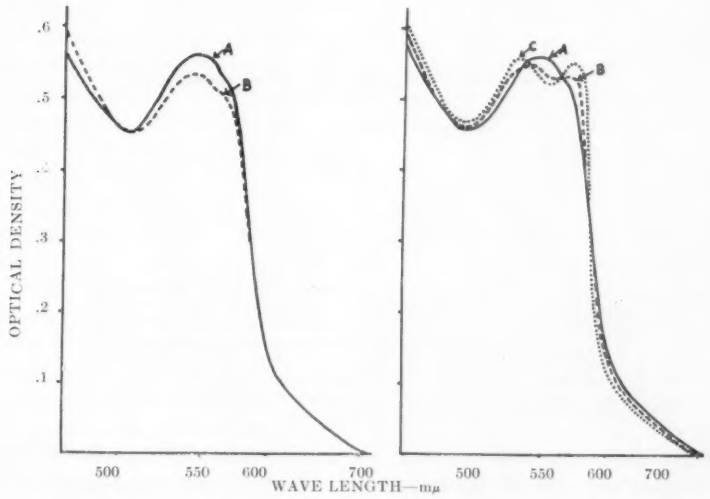

Fig. 2.—(*a*) Curve *A*, spectrum of nodule extract under He; Curve *B*, spectrum of the same extract under scrubbed N_2 5 minutes after the gas change.
(*b*) Curve *A*, spectrum of extract under He; Curves *B* and *C*, extract under He containing 8 ppm and 20 ppm O_2, respectively, 5 minutes after the gas change.

As is illustrated in Figure 2, the use of scrubbed He, to which traces of air had been added, to replace the scrubbed He over the extracts in the cuvettes, showed, that in the first 10 minutes after changing the gas, the hemoglobin reacted with the oxygen to form oxyhemoglobin and not hemiglobin, as shown by the development of peaks at 560–565 mμ and 540 mμ, (Sternberg and Virtanen[4]).

No activity with respect to N_2 could be detected in extracts from which the sonic step had been omitted, and sonic treatment for 20 min. also produced inactive preparations. All extracts, however, contained hemiglobin after standing for 4 hours under atmospheres containing traces of added air, irrespective of presence or absence of activity with N_2.

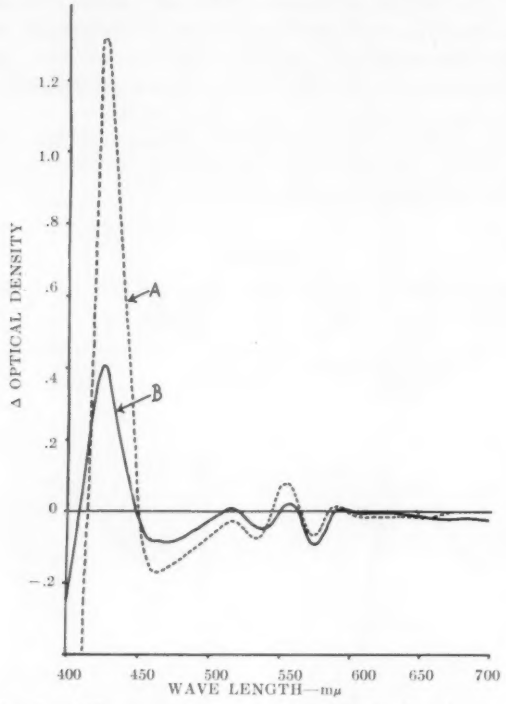

FIG. 3.—Difference spectra (reduced-oxidized) of soybean nodule extracts. *A*, oxidized with air and reduced with dithionite. *B*, oxidized with air reduced with bacteroids under an atmosphere of He. Both cuvettes contained 2.8 ml nodule extract and 0.2 ml of bacteroid suspension; the reference cuvette was bubbled with air between readings and the sample cuvette was filled with He. The baseline is the difference spectrum before addition of dithionite for *A*, and change of air to He in *B*.

When washed bacteroids were added to an air oxidized preparation from which air had been replaced by He the hemiglobin was rapidly reduced as shown in Figure 3. Preparations capable of showing oxidation of hemoglobin with N_2 failed to do so when washed bacteroids were added from the Thunberg cap prior to the change in the gas atmosphere. Thus, bacteroids are capable of reducing hemiglobin to hemoglobin and of reversing the oxidation produced by N_2 in active preparations.

Discussion.—The observations herein reported add to the arguments of Hamilton, Shug, and Wilson[2] for the validity of the N_2 effect on sonic extracts of soybean nodules. These workers could detect no O_2 in their N_2 samples in the mass spectrometer and concluded from the specificity of the reactions with H_2, He and N_2, that O_2 was not involved in the oxidation observed with N_2. The observation that traces of O_2 as low as 8 p.p.m. in He, produced oxygenation of the hemoglobin in the preparations within the same limits of time in which N_2 produced oxidation of the pigment, support these conclusions. The absence of activity from extracts not exposed to the sonic treatment and from extracts subjected to 20 minutes of sonic treatment, lends support to the conclusion that active preparations contain fragments of the nitrogen fixing complex. These fragments are capable of binding nitrogen and accepting hydrogen from hemoglobin. The observations of Hamilton, Shug, and Wilson[2] that H_2 produces the reverse effect, reducing hemiglobin to hemoglobin may mean, as suggested by Winfield,[5] that hydrogenase in nodules acts as a hydrogen transferase between a hydrogen carrier and the activated bound N_2, and may in fact be part of the nitrogen fixing complex.

The observation reported by Appleby and Bergersen[6] that bacteroids could reduce hemiglobin under anaerobic conditions have been extended here to show a possible role of hemoglobin as a coupling agent between the terminal respiratory pathways of the bacteroids and the nitrogen-fixing site.

Bergersen and Briggs[7] demonstrated a system of membrane envelopes, within the host cytoplasm, enclosing groups of bacteroids. From cytochemical tests for heme iron, it appears that hemoglobin in soybean nodules is located within these membrane envelopes, (Bergersen, unpublished). Bacteroids are capable of high rates of O_2 uptake (Bergersen[3]) and since the dimensions of the envelope-system indicates about 10^4 envelopes per host cell, each containing at least four bacteroids, O_2 tension would appear to be very low indeed. Under these conditions it is possible that the hemoglobin becomes an extracellular part of the terminal respiratory pathway of the bacteroids, which in nitrogen fixing nodules, may well terminate with the reduction of atmospheric nitrogen. This type of pathway has already been suggested by Parker,[8] and may be summarized as follows: bacteroid respiration→hemoglobin→nitrogen-fixing complex.

The use by Hamilton, Shug, and Wilson[2] of nodules frozen under H_2 for the preparation of active extracts could not be repeated. Only extracts prepared from freshly harvested, actively N_2-fixing nodules were capable of producing hemoglobin oxidation in the presence of N_2, although some frozen nodule preparations showed shifts of absorption spectra in the presence of H_2. It is well known that nodules cease to fix nitrogen after such treatments as freezing and this again tends to support the specificity of the reactions under discussion.

Summary.—The ability of sonic extracts of fresh, active soybean nodules to react with gaseous N_2 to produce oxidation of hemoglobin in the preparation, has been confirmed, and the reaction has been shown to be complete within 5 minutes. The possible role of traces of contaminating O_2 in the N_2 in producing the effect has been eliminated by the demonstration that traces of O_2 first cause measurable oxygenation, and not oxidation of the hemoglobin. Oxidation of the pigment in the preparation follows after standing for several hours.

The ability of bacteroids to reduce oxidized hemoglobin has been demonstrated

and the possible role of the pigment as a hydrogen carrier between the terminal respiratory pathway of the bacteroids and the nitrogen-fixing complex is discussed.

* This research was supported in part by grants from the Atomic Energy Commission Contract AT (11-1)-64, and from the National Institutes of Health Grant E 1417 (C5) while one of the authors (F. J. B.) was on study leave from the Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, Canberra, A. C. T. Australia.

[1] Shug, A. L., P. B. Hamilton, and P. W. Wilson, in *Inorganic Nitrogen Metabolism* (Baltimore, Johns Hopkins Press, 1956) Chap. IV, pp. 316–363.

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ERRATA

In the paper "Optical, Electromagnetic, and Satellite Observations of High-altitude Nuclear Detonations—Part I," appearing in these PROCEEDINGS, **45,** 1208–1221 (1959), the two sentences beginning on line 22 of page 1215 should read as follows:

"A height of 100 km for the aurora at a range of 800 km requires an elevation angle of 3° 20′, apparently below the visual aurora. In this case, the ground conjugate point is deviated by about 200 km to the northwest of the conjugate point for event III computed at the RAND Corporation by Vestine and Karzas."

The reference number on line 9 of page 1215 should be 2, not 13.

PHILIP NEWMAN

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